

Methods for assessing the adhesion of probiotic and canine gut-derived lactic acid producing bacteria to the canine intestinal mucosa *in vitro* and measuring mucosal secretory IgA

by

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ACADEMIC DISSERTATION

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1. ABSTRACT

The intestinal microbiota is a complex ecosystem consisting of numerous species of bacteria. Indigenous intestinal microbiota is considered an integral part of the host's defence mechanisms. It forms a barrier against pathogen colonization and also influences the host's immunological, biochemical and physiological features. The resident indigenous microbiota includes several species of lactic acid bacteria (LAB), which have an important protective function in the gut.

Probiotics, defined as “microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host”, are usually a species of LAB. Selected probiotic LAB have been shown to elicit beneficial health effects in humans and in murine models. The potential positive effects on dogs' health have not been extensively examined and there are no established guidelines for evaluating these effects of canine probiotic LAB.

The present study aimed to develop a method to measure intestinal secretory immunoglobulin A (sIgA) in dogs, and to assess the suitability of various *in vitro* methods utilized in human probiotic studies in veterinary probiotic evaluation. Study I applied the *in vitro* intestinal mucus model utilized in human probiotic studies to canine use, utilizing jejunal chyme from permanently fistulated dogs. The intestinal mucus model was employed also in Study II, when the competitive exclusion of pathogenic bacteria was investigated, and again in Study III, which focused on the species specificity

of LAB adhesion. This model was found to be suitable in canine *in vitro* adhesion studies.

Competitive exclusion of pathogens is one of the most important beneficial health claims of probiotic bacteria. The competitive exclusion study (Study II) suggests that certain probiotic LAB reduce the *in vitro* mucus adhesion of the canine intestinal pathogen, *Clostridium perfringens*. However, increased *in vitro* mucus adhesion of *Campylobacter jejuni* caused by the tested enterococci may be a cause for concern.

Adhesion of probiotic LAB has earlier been shown to be host specific. Host specificity has been considered to be essential for probiotic LAB to exert their beneficial health effects. This is why it is recommended as one of the selection criteria for probiotic LAB. Results from Study III showed the adhesion properties to be LAB strain dependent rather than animal species dependent.

In humans and experimental rodent models, probiotic LAB have been shown to improve the intestine's immunological barrier, particularly immunoglobulin A (IgA) responses. Serum IgA (S-IgA) or fecal sIgA are not considered to be reliable when assessing intestinal IgA responses. Study IV developed a novel method to measure mucosal IgA in duodenal brush samples obtained via endoscopy. A marked variance in sIgA concentrations was measured from different sites of duodenal mucosa. Moreover, it can be concluded that neither S-IgA nor salivary sIgA are suitable parameters for the assessment of duodenal sIgA competence in dogs.

2. ABBREVIATIONS

CFU	Colony forming units
ELISA	Enzyme-linked immunosorbent assay
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
HEPES	N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid
HH	HEPES-Hanks buffer
Ig	Immunoglobulin
LGG	<i>Lactobacillus rhamnosus</i> GG
OD	Optic density
MRS	De Man, Rogosa, Sharpe broth
O/N	Over-night
sIgA	Secretory immunoglobulin A
S-IgA	Serum immunoglobulin A
SRID	Single radial immunodiffusion
SD	Standard deviation

3. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals (I-IV).

- I Rinkinen, M., Mättö, J., Salminen, S., Westermarck, E., Ouwehand, A.C. (2000).
In vitro adhesion of lactic acid bacteria to canine small intestinal mucus. *Journal of Animal Physiology and Animal Nutrition* 84, 43-47
- II Rinkinen, M., Jalava, K., Westermarck E., Salminen S., Ouwehand, A.C. (2003)
Interaction between probiotic lactic acid bacteria and canine enteric pathogens: a risk factor for intestinal *Enterococcus faecium* colonization?
Veterinary Microbiology 92, 111-119
- III Rinkinen M., Westermarck E., Salminen S., Ouwehand, A.C. (2003) Absence of host specificity for *in vitro* adhesion of probiotic lactic acid bacteria to intestinal mucus. *Veterinary Microbiology* 97, 55-61
- IV Rinkinen, M., Teppo, A. M., Harmoinen, J., Westermarck, E. (2003) Relationship between canine mucosal and serum immunoglobulin A (IgA) concentrations: serum IgA does not assess duodenal secretory IgA.
Microbiology and Immunology 47, 155-159

4. INTRODUCTION

The intestinal microbiota is a complex ecosystem consisting of numerous species of bacteria. Little is known about the composition of canine intestinal microbiota since most of the studies have focused either on humans or rodent models. In humans, it is estimated that there are more than 400 known bacterial species present in the gut (Moore and Holdeman 1974, Berg 1996, Vaughan et al 2000). The actual number of microbes is likely to exceed this figure as the estimation is based on traditional cultivation methods available during the mid-1970s. Total numbers of bacteria amount to as many as 10^{14} cells, forming approximately 95% of all the cells of an individual human or animal. As a result, the intestinal microbiota outnumbers the body's eucaryotic cells (Savage 1977, Blaser and Musser 2001). This system is in a finely tuned equilibrium within itself and in relation to the host (Salminen and Deighton 1992).

In the early 20th century, Elie Metchnikoff, a Russian zoologist, bacteriologist and Nobel laureate, postulated that “friendly” intestinal bacteria contributed to the longevity of the Georgian people. He believed that consuming fermented milk products rich in lactic acid bacteria (LAB) is beneficial for humans as they reduce the number of harmful, “toxin-producing” bacteria in the gut (Metchnikoff 1907). At present, the indigenous intestinal microbiota is considered an integral part of the host defence mechanisms. It forms a barrier against pathogen colonization and also influences the host's immunological, biochemical and physiological features (Tannock 1999). Disturbances in the gut

microbiota may result in diarrhoea, malabsorption and chronic intestinal inflammation (Johnston 1999). Acute diarrhoea may be fatal as pathogens may invade the host's tissues, causing bacteremia and sepsis.

The resident indigenous microbiota includes several species of LAB, which have an important protective function in the gut. To be able to induce a disease, a pathogen must first colonize the intestinal mucosa. In order to do this, it must first overcome the host defence mechanisms (e.g. gastric acidity, bile acids, gut peristalsis, secretory immunoglobulin A), and beat the established mucosal microbiota in the competition for nutrients and suitable adhesion sites on the gut mucosa. LAB are documented to have a marked role in pathogen prevention in the intestinal ecosystem. In humans, the best-documented health effect of many specific probiotics has been a reduction in the risk of acute gastroenteritis or a shortening of the duration of viral or bacterial diarrhoea (For reviews see: Salminen and Deighton 1992, Guandalini et al 2000, Alvarez-Olmos and Oberhelman 2001, Reid and Burton 2001, Tagg and Dierksen 2003).

Adhesion to the intestinal mucosa is considered one of the main mechanisms by which probiotic LAB exert their beneficial health effects. It is regarded important for transient colonization (Alander et al 1999), enhanced healing of the damaged gastric mucosa (Elliott et al 1998), modulation of the immune system (Schiffrin et al 1997, O'Halloran et al 1998, Perdigon et al 2002) and antagonism against pathogens (Jin et al 2000). Many studies have examined the adhesion properties of probiotic LAB. As mucosal adhesion is very difficult to study *in vivo*, several *in vitro* methods have been developed. Samples of

intestinal mucosa, epithelial cells and mucus can be used for adhesion assay (Tuomola 1999c).

Probiotics are being widely utilized in farm animal husbandry to improve breeding performance in stressful conditions such as high animal concentration, early weaning or rapid growth (Fuller, 1989). Probiotics are also considered beneficial for canine health in similar situations, although scientific evidence from controlled trials is very scarce (Barrows and Deam 1985, Biourge et al 1998, Pasupathy et al 2001, Benyacoub et al 2003, Vahjen and Männer 2003). For admission to the European common market, probiotics intended for animals have to be tested according to the Feed Additive Directive 70/524/EC. However, it has been evident that there are currently no criteria available to assess their effectiveness (Lahrssen and Zentek 2002).

As adhesion to intestinal tissue is one of the cornerstones of probiotic function, it is therefore regarded as one of the main selection criteria for probiotic LAB. Other criteria include an ability to survive gastric acid and bile, activity against pathogens and mucosal immunomodulation (Saarela et al 2000, Dunne et al 2001). At present, these criteria are applied only to probiotics intended for human consumption. Such guidelines would also benefit research and development in the veterinary probiotic field. Demand for appropriate strain identification and nomenclature; clinically evaluated and reported health effects; documented ability for the strain to survive and temporarily colonize the host, and safety for both animals and humans are applicable also to probiotics intended for animal consumption.

5. REVIEW OF THE LITERATURE

5.1 Lactic acid bacteria and probiotics

Lactic acid bacteria (LAB) are gram-positive, aerotolerant, catalase negative rods or cocci producing lactic acid as their main fermentation product. They form a heterogeneous group of bacteria, the genera of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella* being the best known. Most LAB are non-pathogenic and they are associated with wide variety of sources, such as plant material and various foods (Axelsson 1998). They also form a substantial part of the intestinal microbiota and are believed to have a major effect on host's well-being (Vaughan et al 2002). The knowledge of the canine intestinal LAB is scarce, as only few studies have addressed the canine intestinal microbiome, including LAB (Smith 1965, Clapper 1970, Davis et al 1977, Benno et al 1992, Greetham et al 2002). In addition, many of these studies date back to times when molecular techniques were not available, and LAB were not identified to species level. Also the classification and nomenclature of LAB has been subjected to various changes during recent years. In a recent study, fecal microbiota of four Labrador retrievers was examined and *S. bovis* and *L. murinus* were found to be the most prevalent culturable LAB species (Greetham et al 2002).

Bifidobacteria, although they share much similarity with LAB, are strictly speaking not LAB because of the different metabolic path way by which they produce lactic acid; the fructose-6-phosphate pathway (Ballongue 1998)

The word “probiotic” originates from the Greek word meaning “for life”. Probiotics are defined as “microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host” (Salminen et al 1999).

Probiotics are most often of lactic acid bacteria (LAB); bacteria belonging to other genera (e.g. *Bacillus*) and yeasts (e.g. *Saccharomyces*) have also been used.

The term "probiotic" was first used in its current meaning in the mid-1970s (Parker 1974). The term was again revised by Fuller (1989), who defined it as a “live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance”. Later, this definition was broadened to cover humans (Havenaar and Huis in’t Veld 1992) and specifically probiotic functional foods (Salminen et al 1998).

Although probiotics were mostly used on domestic animals before they began to be successfully used in human health foods, little is known about their beneficial health effects on dogs. Apart from this, there are no generally accepted guidelines available to assess the *in vivo* or *in vitro* effectiveness of potential probiotic bacteria intended for pet animal use. Despite this, probiotics are effectively marketed to veterinarians and dog owners and are becoming increasingly popular also in promoting canine health.

5.1.1 Beneficial documented health effects related to probiotics intended for human consumption

Selected probiotic LAB have been shown to elicit beneficial health effects in humans (Salminen et al 1998, Sanders 2003), Table 1. Some of these health claims are better scientifically proven than others: for example probiotic *L. rhamnosus* GG was able to reduce the prevalence of atopic eczema by 50% in a placebo-controlled study. In addition, several studies have documented that selected probiotics reduce the duration of rotavirus diarrhea in children. However, many other probiotics failed to shorten the duration of rotavirus diarrhea, suggesting that this favourable effect is dependent on the probiotic strain. Probiotics may prevent travellers' diarrhea, although this claim is not strongly supported by the present data. Ability to prevent urinary tract infections, and to reduce cholesterol are also listed amongst beneficial health effects, though scientific evidence for these claims is rather weak (Ouwehand and Vesterlund 2003). In conclusion, some of the beneficial health effects are better documented and therefore widely accepted (e.g. prevention of allergies in infants; shortening the duration of rotavirus diarrhea), whereas more studies are needed for some other claims (e.g. lowering of serum cholesterol; prevention of urinary tract infections). In addition, positive health effects may be strain-dependent, and cannot therefore be extrapolated to other probiotic bacteria. Also in murine models probiotics have been documented as having several positive health effects (For more complete reviews see Erickson and Hubbard 2000, Macfarlane and Cummings 2002, Guarner and Malagelada 2003).

The possible effects on dogs' health have not been extensively examined, even though probiotics are frequently marketed for canine use as well.

Alleviation and prevention of allergies	Down-regulation of inflammatory responses in
Reduction of cariogenicity	inflammatory bowel disease
Reduction of carcinogenicity and mutagenity	Antimicrobial activity in small bowel bacterial
Cholesterol reduction	overgrowth
Immunomodulation	Prevention of kidney stones by altering the
Alleviation of lactose intolerance	gut flora influencing oxalate degradation
Prevention of vaginosis and urinary tract	Prevention and alleviation of antibiotic-
infection by antipathogen activity	associated, rotavirus and travellers'
Alleviation of hypertension	diarrhoea

Table 1. Documented beneficial health effects in controlled human studies. Modified from Sanders, 2003.

5.1.2 Beneficial documented health effects in dogs

There are only a few reports of beneficial health effects in dogs. *Enterococcus faecium* SF68 has been documented as enhancing specific immunological responses in young dogs (Benyacoub et al 2003) and *E. faecalis* FK-23 stimulated non-specific immune functions in healthy adult dogs (Kanasugi et al 1997). Pasupathy and co-workers (2001)

evaluated the effect of *Lactobacillus acidophilus* on the digestibility of food and growth of puppies. They concluded that *Lactobacillus* supplementation has a favourable effect during the active growth period, although differences between the study group and control group were not significant.

5.1.3 Adhesion to intestinal mucosa

The mechanisms used by pathogenic bacteria to attach themselves to the intestinal mucosa have been broadly examined in order to understand and prevent enteric bacterial infections (Mouricourt 1997, Edwards and Puente 1998). The nonpathogenic indigenous microbiota in the gut has received much less attention and the exact mechanisms by which LAB bind to intestinal mucosa have yet to be clarified. Adhesion to the intestinal mucosa, followed by at least transient colonization is considered necessary for probiotic LAB to exert their favourable effects as it prolongs the contact period with the host, thus allowing more time for the probiotic to exert its beneficial health effects. Adhesion to mucosa is regarded as important for transient colonization (Alander et al 1999) enhanced healing of the damaged gastric mucosa (Elliott et al 1998), modulation of the immune system (Schiffrin et al 1997, O'Halloran et al 1998, Perdigon et al 2002) and antagonism against pathogens (Jin et al 2000, Hirano et al 2003).

Mucosal colonization with non-pathogenic resident microbiota is of particular importance for the protection of the host against pathogenic invaders. This is best illustrated in

animals without normal intestinal microbiota, which considerably are more susceptible to infections (Baba et al 1991).

In a rodent model, colonization of probiotic *Bifidobacterium infantis* 1222 was reported to prevent the intestinal wall colonization of pathogenic *Bacteroides vulgatus*, one of the intestinal commensal pathogens considered to play a role in the development of human inflammatory bowel disease (IBD). *B. infantis* prevented *Bacteroides* from invading the gut epithelium, which could have led to increased systemic antibody responses and the development of IBD (Shiba et al 2003).

Lactobacilli able to colonize the gut mucosa also seem to have a potential to affect the intestinal immune functions, and ability to colonize the gut mucosa is regarded as a valuable selection criteria for immunomodulation (Herias et al 1999, Schiffrin et al 1997).

5.1.3.1 Non-specific and specific bacterial adhesion

In general, bacterial adhesion can be divided into two parts: non-specific and specific adhesion.

Non-specific binding of bacteria is based on van der Waals and electrostatic forces between the cell and the mucosal surface, explained by the so-called DLVO-theory. This theory postulates that although bacteria are negatively charged, they are still attracted to negatively charged host tissue because the attracting van der Waals forces are stronger

than the electrostatic repulsion. However, the DLVO-theory originally described the attachment of inert particles to a solid substratum but has also been applied to bacteria (Busscher and Weerkamp 1987). It appears that the interactions between living bacteria and mucosa are much more complicated to be explained by the DLVO-theory alone (Tuomola 1999c). An additional explanation is based on the hydrophobic molecules on the bacterial surface, which counteract the repulsive electrostatic forces, allowing bacteria to draw near the negatively charged mucosal. This type of an adhesion is considered to be weak and reversible (Ofek and Beachey 1995, Vigeant et al 2002). Non-specific bridging between a bacterium and substratum may also be due to hydrophobic interactions or hydrogen bonding (An et al 2000).

Specific adhesion has been described as “lock and key” interaction between bacteria and the host mucosa and is mediated by bacterial adhesins and corresponding receptors in the mucosal epithelium (An et al 2000, Dunne 2002). The matching connection between receptor and adhesin allow numerous bonds between the bacterium and host cell. The binding thus formed is much stronger when compared to non-specific adhesion, as it is not likely for all the adhesive bonds to rupture simultaneously. Adhesion can be inhibited by altering the structure of adhesins or receptors with enzymes or other chemical compounds; or with antibodies mimicking the receptor or adhesin (Beachey 1981). Probiotic LAB may employ both types of mechanisms when competing for survival in the intestinal mucosa, for example *L. crispatus* was reported to inhibit the *in vitro* adhesion of *E. faecalis* due to the combined effect of both bactericidal activity and competition for attachment site (Todoriki et al 2001).

Bacterial lectin adhesins can be located on cell surface appendages, as in tips of the fimbriae in *E. coli* (Thomas et al 2002). LAB and bifidobacteria do not commonly possess such prominent structures; although fimbriae and flagella have been identified in some vaginal LAB strains (McGroarty 1994) and *Bifidobacterium longum* (Schell et al 2002).

Bacteria belonging to *L. acidophilus* group are documented to have lectin-like proteins in the surface layer protein (Yamada et al 1994). Such elements in the surface layer protein may contribute to the bacterial adhesion, as they bind to carbohydrate portions of the intestinal mucosal layer (Matsumura et al 1999). In the intestine, the mucus layer covering the gut epithelium is rich in glycoproteins and glycolipids, providing abundantly carbohydrate moieties for bacterial adhesion (Beachey 1981). The exact mechanisms of the lactic acid producing bacteria adhesion in the intestinal mucosa are not very well characterized. However, *L. fermentum* was documented to bind to mucus glycoproteins isolated from porcine gastric mucus (Henriksson et al 1996), and several lactic acid producing bacteria adhered to human ileostomy glycoproteins (Tuomola et al 1999a). These findings suggest that glycoproteins in intestinal mucus can act as suitable receptors.

Bacterial adhesion is of special importance in the small intestine, where the intestinal peristalsis may detach the bacteria from the mucosal wall. Stressful conditions in the

small intestine caused by luminal flow may alter the function of known adhesive proteins and may actually enhance the bacterial binding (Isberg and Barnes 2002).

Adhesive structures may also be involved in the immunostimulation triggered by LAB, e.g. *L. casei* strain CRL 431 capable of immunostimulation was found to have protruding cell surface structures identified as lectins (Morata de Ambrosini et al 1998).

5.1.4 Host specificity of probiotic LAB

Adhesion of probiotic LAB has been reported to be species specific (Fuller 1973, Barrow et al 1980, Mäyrä-Mäkinen et al 1983). Adhesion and at least temporary colonization to host tissue is regarded as important factor for the probiotic to induce its beneficial health effects. Therefore, host specificity has been considered to be a desirable property for probiotic bacteria. It is thus recommended as one of the selection criteria (Salminen et al 1998, Saarela et al 2000). Despite this criterion, none of the probiotics evaluated in the canine studies were of dog origin. However, the demand for species specificity has been discussed, although many of the probiotics with most scientific data are of human origin (Salminen et al 1998). On the other hand, LAB of human origin have shown good adhesive and immunomodulatory properties in rodents (Wagner et al 1997, Dieleman et al 2003), and in fish (Nikoskelainen et al 2003).

Even though the importance of host species specificity has been debated, no studies have focused on interspecies differences when it comes to the adherence properties of

probiotic LAB adhesion although many probiotics – especially products intended for animal use - are marketed for more than one species (Anon 2002).

It has been shown that LAB populations in experimental animals (mice and rats) are not dependent on genetics but rather are influenced by environmental factors. Such findings underline the effect of intestinal microbiota for studies in probiotic trials and a similar competitive role for faecal bacteria has been reported in adherence studies using Caco-2 cells (de Waard et al 2002, Haller et al 2001). The specificity of adhesion properties should thus be further clarified prior to using host specificity as a key for selection procedures.

5.1.5 LAB adhesion to intestinal mucosa

5.1.5.1 Structure of intestinal mucosa and mucus production

The intestinal lumen is covered by mucous membrane. Its surface epithelium consists of a single layer of columnar cells called enterocytes. Scattered between these cells are specialized enterocytes, goblet cells, which synthesize and excrete mucus. Mucus acts as a lubricant and a protective layer on top of the enterocytes. The production of mucus is a constant process as it is lost in faeces and by bacterial degradation (Schummer and Nickel 1979, Neutra and Forstner 1987).

Mucus consists mainly of water (up to 95%) and mucus glycoproteins (mucins; up to 5%) that dictate the viscoelastic characteristics of mucus. Additionally, lipids, immunoglobulins and salts are present in the intestinal mucus gel (Neutra and Forstner 1987). Mucins are macromolecules with a peptide core linked to oligosaccharide chains through O-glycosidic bonds. Oligosaccharides consist of N-acetyl or N-glycolyl neuramine acids, fucose, galactose, N-acetyl glucosamine and N-acetyl galactosamine. The sugar composition of mucins varies depending on the host animal, blood group and anatomical location. E.g. *Bacteroides thetaiotaomicron* can also induce the host to add in particular fucose to the mucins (Xu and Gordon 2003).

In addition, intestinal bacteria degrade mucins differently, so the composition of mucus cannot be regarded as uniform in all species, and varies also along the intestinal tract (Forstner 1978). The composition of canine intestinal mucus has not been examined, but homologies are reported between canine tracheal mucin glycoproteins and human tracheobronchial and intestinal mucin glycoproteins (Verma and Davidson 1993). The intestinal mucus layer is also an important part of the host's defence mechanisms, protecting the underlying epithelium from bacterial and environmental antigens, mechanical damage and digestive enzymes (Deplancke and Gaskins 2001).

Most probiotic adhesion studies have focused on enterocyte adhesion (Salminen et al 1998). However, mucus covering the enterocytes is the first contact surface for intestinal bacteria in the gastrointestinal tract and an important ecological niche for bacterial adhesion and potentially also for competitive exclusion (Neutra and Forstner 1987,

Mikelsaar et al 1998). Mucins can be divided histochemically into neutral and acidic mucins. Acidic mucins are further classified as sialomucins or sulphomucins (Niv et al 1996). Several mucin genes have been identified in humans (Buisine et al 2001). No studies exist on the characterization of canine intestinal mucins or mucin genes.

5.1.5.2 Methods for *in vitro* adhesion studies

LAB adhesion is difficult to study *in vivo*, which is why several *in vitro* methods have been developed. In general, when using *in vitro* assays the bacteria are first incubated with the substratum to allow the bacterial cells to bind to it. After the bacteria have adhered, the unbound bacterial cells are removed and the adhered bacteria enumerated (Tuomola 1999c).

Several methods have been utilized to investigate the *in vitro* adhesion of probiotic bacteria. Samples of intestinal mucosa, epithelial cells and mucus can be used for adhesion assay. Intestinal tissue samples can be obtained from animals *post mortem* or during celiotomy. However, as this may have a certain ethical burden, also less invasive *in vitro* models simulating the gut mucosa have been developed for bacterial adhesion studies. Different *in vitro* methods have been used for human probiotic characterization. Conway and co-workers (1987) used ileal cells from ileostoma patients in their lactobacilli adhesion studies. Tissue culture lines of human intestinal origin are widely used for bacterial adhesion assays. Commercial cell lines such as Caco-2 and HT-29 are readily available and, as similar techniques are utilized, make the results of independent study groups more comparable (Tuomola 1999c). The lack of commercially available

canine intestinal cell lines hinders this kind of uniform research of intestinal bacterial adhesion in dogs.

In vitro evaluation of LAB adhesion to intestinal mucus extracted from human faeces has proved to be a suitable model for both studying probiotic adhesion and offering an additional indicator of adhesion properties (Kirjavainen et al 1998). *In vitro* adhesion models utilizing both Caco-2 cell cultures and human ileostomy glycoproteins have been compared. The adhesive properties of the investigated LAB were found to vary, although some strains showed similar adhesion traits. In general, LAB tended to adhere to Caco-2 cells more readily than to the intestinal glycoproteins (Tuomola 1999a). The method has also been used to investigate LAB adhesion in pigs (Blomberg et al 1993) and fish (Nikoskelainen et al 2001a).

5.1.5.3 Effect of jejunal chyme on *in vitro* probiotic adhesion

The canine gastrointestinal tract is constantly challenged by numerous microbial threats that may be harmful to the host. Therefore several defence mechanisms in the gut work to prevent hostile invaders from entering through the intestinal wall and infecting the animal. Secretory IgA (sIgA) has an important role as a first line of defence against bacteria entering the intestine. It binds microbial antigens and in this way acts as an “antimicrobial paint” that prevents bacteria from adhering to the mucosal surface, thus protecting the organism from pathogen colonization and possible invasion (van Egmond 2001). In addition to sIgA, there are also other antibacterial substances present in the gut

lumen and mucosa: bile acids and pancreatic enzymes have a notable role in guarding the organism against pathogenic bacteria (Schiffrin and Blum 2002). Also other members of the innate immunity (e.g. lysozyme, lactoferrin, defensins, antibacterial peptides and secretory phospholipase A) are present in the intestinal lumen (Pitman and Blumberg, 2000).

Probiotic bacteria have to survive these hostile conditions in the stomach and proximal duodenum before they reach their potential colonization sites, the small and large bowel. In the upper gastrointestinal tract digestive enzymes, intraluminal antibacterial secretions and sIgA will try to interfere with the adhesion and colonization of engulfed bacteria. It is vital for a probiotic to be able to survive these defensive mechanisms and still be able to maintain its adhesive properties. Resistance to bile is generally regarded as an important trait when evaluating the probiotic potential of LAB (Chou and Weimer 1999). This can be mimicked *in vitro* by assessing the ability of probiotic bacteria to grow in the presence of bile (Dunne et al 2001). However, as bile is not the sole antibacterial factor in the gut, it may be relevant to expect other elements in the intestinal chyme to have a considerable effect on bacterial adhesion, too. Little is known about the synergy between different antimicrobial factors in the intestinal chyme. It might therefore be more appropriate to incubate bacteria with intestinal chyme instead of pure bile in order to simulate digestion and study its effects on adhesion when evaluating the *in vitro* properties of potential probiotics.

5.1.5.4 Competitive exclusion of intestinal pathogens by LAB

Competitive exclusion of pathogens is thought to be one of the most important beneficial mechanisms of probiotic bacteria (Adlerberth 2000, Rolfe 2000, Reid and Burton 2002). Competitive exclusion by intestinal bacteria is based on bacteria-to-bacteria interaction mediated by competition for available nutrients and mucosal adhesion sites. In order to gain a competitive advantage, bacteria can also modify their environment to make it less suitable for their competitors. The production of antimicrobial substances, such as lactic and acetic acid, is one example of this kind of environmental modification (Schiffrin and Blum 2002).

To be able to hinder pathogen colonization (and possible subsequent invasion), probiotics are believed to have several pathways. They can reduce the viability of a pathogen by producing noxious substances, such as lactic acid in the case of *L. casei* and *L. acidophilus* against EHEC (Ogawa et al 2001); or non-acidic material, e.g. bacteriocins, like *L. acidophilus* that was reported to suppress the growth of *Salmonella* Typhimurium, EHEC and *Shigella flexneri* (Coconnier et al 1993).

Another way to prevent pathogen colonization is to interfere with their adhesion on the mucosal receptors. For example, *L. reuteri* and *L. crispatus* competed with the receptor sites on the host cell with *Salmonella* Typhimurium and enterotoxigenic *E. coli* (Todoriki et al 2001); *L. reuteri* was also effective in preventing the *in vitro* binding of *Helicobacter pylori* to host cell receptors (Mukai et al 2002).

The effect of probiotic LAB on the competitive exclusion of pathogens has been demonstrated using human mucosal material *in vitro* (Tuomola et al 1999a, Hirano et al 2003), and *in vivo* in chickens (Hirn et al 1992) and pigs (Genovese et al 2000). Hirano and colleagues (2003) showed, that the well-adhering strain *L. rhamnosus* was capable to inhibit the internalization of Enterohemorrhagic *E. coli* (EHEC) to a human intestinal cell line *in vitro*. The result suggests that a close interaction with the host cells may have been responsible for this suppression of EHEC internalization. In Finland, the competitive exclusion method has effectively reduced the incidence of salmonellae in broiler chicks by (Hirn et al 1992).

As there is emerging evidence of the development and spreading of antimicrobial resistance amongst pathogenic bacteria, every attempt to reduce this risk is welcome. The use of probiotic bacteria instead of antibiotics is an intriguing prospect in the treatment of acute and chronic intestinal disorders, both in humans and animals. This concept has been recommended by the World Health Organization (WHO) as an alternative to antibiotics for the prevention and control of production-related animal diseases (WHO 1997).

If specific probiotics can be proven to possess antipathogenic effects, this could reduce the use of antimicrobial substances also in canine medicine, thus contributing to a lowered risk of spreading antibiotic resistance amongst pathogenic bacteria.

Antibiotics often interfere with the natural homeostasis in the intestinal tract. This may lead to antibiotic-induced diarrhoea and possibly even to severe diarrhoea and colitis caused by an overgrowth of resistant opportunistic enteric pathogens (Wiström et al

2001). Specific probiotics have been shown to be a promising option when treating certain antibiotic-responsive intestinal disorders in humans (Cremonini et al 2002). Probiotics are also marketed for administration to antibiotic-medicated dogs in order to lessen GI-tract side effects such as diarrhoea (Anon 2002). However, no published reports of controlled trials confirm this concept in dogs.

5.2 Evaluation of the effects of probiotic LAB on intestinal sIgA production

5.2.1 Mucosal IgA

IgA is the predominant immunoglobulin of mucosal surfaces (Goldblum 1990, Ginel et al 1993, Guilford 1996). Most canine IgA is sIgA, which is produced by mucosal lymphocytes (Goldblum 1990, Ginel et al 1993, Heremans 1974, Mestecky et al 1999). Dimeric IgA is attached to the secretory component on the basolateral side of enterocytes, and the complex (sIgA) is transported and released onto the mucosal surface (Toy and Mayer 1996, Mestecky et al 1999). In the gut, sIgA plays an important role in the intestines' first-line defence against enteric antigens (e.g. bacteria, toxins, viruses and dietary antigens). Compared with other immunoglobulins, sIgA is more resistant to proteolytic enzymes and does not elicit an inflammatory response (Mestecky et al 1999, Isolauri et al 2001). In humans, IgA is divided into two subclasses, IgA₁ and IgA₂, the latter being produced mainly in the lower intestinal tract and being resistant to bacterial proteases (Mestecky et al 1999). Similar subclasses have not been identified in dogs. Important features of IgA include anti-inflammatory and immune-regulating activities (Goldblum 1990, Isolauri et al 2001).

Relative IgA deficiency is the most common primary immunodeficiency in man and a similar condition has also been attributed to dogs (Ginel et al 1993). In dogs, IgA deficiency has been associated with increased susceptibility to infections such as small intestinal bacterial overgrowth (SIBO) and inflammatory bowel disease (IBD) (Felsburg et al 1985, Batt et al 1991, Campbell et al 1991, German et al 1998).

5.2.2 Assessing the mucosal IgA status in dogs

Serum immunoglobulin A (S-IgA) concentration measurement is broadly used when assessing canine immunocompetence (German et al 1998). As most canine S-IgA is dimeric and synthesized in gut-associated lymphoid tissue, it is believed that in dogs S-IgA reflects IgA production in submucosal plasma cells (Heremans 1974). It has therefore been suggested that low S-IgA also indicates low gastrointestinal IgA concentration (Whitbread et al 1984). German shepherd dogs with low S-IgA also had decreased IgA concentration in the duodenal juice, implying a correlation between S-IgA and mucosal sIgA (Batt et al 1991). However, this concept has been questioned by German and colleagues (1998) who concluded that the serum immunoglobulin concentrations are poor indicators of mucosal Ig secretion in dogs.

Also indices other than S-IgA measurement have been utilized to evaluate intestinal IgA status. The quantity of IgA producing plasma cells has been assessed in intestinal biopsies by immunohistochemical methods (Guilford 1996). Nonetheless, as the

secretory mechanism may be defective in some dogs, the amount of IgA-containing plasma cells does not reliably correlate with the amount of mucosal sIgA in gut (Batt et al 1991).

As both intestines and buccal mucosa are part of the common mucosal immune system, it can be hypothesized that salivary sIgA concentration reflects the intestinal sIgA level (Mestecky 1993). Saliva samples are easy to obtain and cause no inconvenience to the dog. Also faecal IgA concentration measurement has been suggested as an evaluation tool for intestinal immunocompetence. However, this method has been criticized as not representing the true status of the gut's humoral immune system (Ferguson et al 1995).

Measuring the intestinal sIgA concentration in dogs suffering from chronic enteropathies may provide more information about their immunological status. A method assessing intestinal sIgA concentration would be useful when examining patients with suspected immunodeficiencies. The method should be reliable and allow serial measurements and cause minimal discomfort to the dog.

5.2.3 The effect of probiotic LAB on intestinal sIgA responses

In humans and experimental rodent models, probiotic LAB have shown to improve the intestine's immunological barrier, particularly IgA responses (For reviews see: Yasui et al 1999, Gorbach 2000, Isolauri et al 2001). Feeding *Enterococcus faecium* SF68 supplemented food to young dogs has been documented as increasing faecal IgA

concentration, thus implying an improvement in local protective immune response in the gut (Benyacoub et al 2003). However, as immunological tests on faecal samples have been reported as not characterizing the true status of the gut's humoral immune system (Ferguson et al 1995), another method of evaluating the intestinal sIgA level is needed to investigate the potential probiotic-induced effects on local sIgA production in the canine gut.

6. AIMS OF THE STUDY

The aims of the current study were to

- I Study the adhesive properties of probiotic and indigenous canine LAB in the intestinal mucus model *in vitro*
 - a) Apply to canine use an *in vitro* intestinal mucus adhesion model used for characterizing human probiotics, utilizing jejunal chyme from permanently fistulated dogs
 - b) Investigate the effect of jejunal chyme on the adhesion of specific probiotics and indigenous canine LAB *in vitro*
 - c) Investigate the effects of specific probiotics and indigenous canine LAB on pathogen exclusion *in vitro*
 - d) Investigate host species specificity of specific probiotics and indigenous canine LAB *in vitro*
- II To develop a method for assessing intestinal sIgA in dogs and to evaluate indirect methods such as salivary or serum IgA assessment as predictors for intestinal sIgA concentration.

7. MATERIALS AND METHODS

In vitro mucus adhesion model was utilized to evaluate the adhesive properties of selected probiotic and canine-derived lactic acid producing bacteria. Intestinal chyme from permanently fistulated beagles was used as a source of mucus. In addition, *in vitro* pathogen exclusion study was performed using the same method. Using the same method, the species specificity of selected lactic acid producing bacteria was examined. In the last study a method for assessing the intestinal sIgA in duodenal samples was developed.

7.1 Animals

Studies I-III were set up to study the adhesion properties of certain commercially available probiotics (both human and veterinary products) and indigenous canine LAB. In addition these studies sought to evaluate whether the *in vitro* mucus adhesion model would be suitable for bifidobacteria and LAB adhesion studies on canine jejunal chyme.

For jejunal chyme sampling, permanent nipple valves for intestinal access were operated in six healthy beagles (five males, one female) in the mid-jejunum, using the method described earlier (Wilsson-Rahmberg and Jonsson 1997). Operations were approved by the ethics committee of the University of Helsinki and performed 10 months prior to the first study. No changes in the dogs' health or gastrointestinal function were noticed as a result of the valve (Harmoinen et al 2001).

For the mucus preparation, a sample of approximately 8 ml of jejunal chyme was obtained via the valve 2 h postprandial and frozen immediately at -70°C.

Study IV was set up to develop a method to assess duodenal sIgA in dogs. The survey included twenty dogs, twelve female and eight male, approved for this study. These dogs were of nine different breeds and had no previous history of chronic gastrointestinal disease and no clinical signs suggestive of any disorder. The dogs were family pets (excluding nine beagles), and the owners submitted their dogs to this study voluntarily. The beagles were laboratory animals. All dogs were over 12 months old.

The use of experimental animals in these studies was approved by the ethics committee of the University of Helsinki.

7.2 Methods

7.2.1. *In vitro* mucus adhesion assays

The *in vitro* mucus adhesion model was used for the bacterial adherence assays. The method has been modified and described by Kirjavainen and colleagues for human probiotic studies (Kirjavainen et al 1998). The method has been widely utilized in human probiotic adhesion studies later (Tuomola 1999c, Ouwehand et al 1999, Juntunen et al 2001), and also in fish (Nikoskelainen et al 2001). It has not been used for canine studies earlier, so one of the aims of the present study was to evaluate whether it would be suitable for canine *in vitro* adhesion studies, too.

7.2.1.1 Mucus glycoproteins extracted from jejunal chyme and feces (I-III)

Mucus was prepared from canine jejunal chyme essentially as described earlier (Kirjavainen et al 1998, Ouwehand et al 1999b). In brief, jejunal chyme was centrifuged at 12 000 x g to remove particulate matter. Mucus was precipitated from the clear supernatants by dual ethanol precipitation and freeze-dried. Equal amounts of mucus from each dog were pooled and a stock suspension of 5 mg ml⁻¹ in HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid)-Hanks buffer (HH; 10 mM HEPES; pH 7.4) was prepared and stored at -20°C until use.

In Study III, mucus was also isolated from the feces of emu, ostrich, possum (three animals from each species), humans (ten individuals), and from the intestines of a rainbow trout as described earlier (Kirjavainen et al 1998; Ouwehand et al 1999; Nikoskelainen et al 2001). In short, mucus was isolated from the feces by extraction and dual ethanol precipitation technique as described by Miller and Hoskins (1981); and from rainbow trout intestines by gently scraping the gut surface, particulate matter was removed by centrifugation at 13 000 x g.

7.2.1.2 *In vitro* adhesion assay (I-III)

The adhesion of the radioactively labelled bacteria to immobilized intestinal mucus was determined as reported previously (Kirjavainen et al 1998, Ouwehand et al 1999b). Briefly, the mucus stocks were thawed and centrifuged to remove any precipitate formed

during storage and diluted in HH to a concentration of 0.5 mg ml⁻¹. The mucus was passively immobilized on polystyrene microtitre plate wells (Nunc Maxisorp, Roskilde, Denmark) by overnight incubation at 4°C. Excess mucus was removed by washing twice with HH. Radiolabelled bacteria were added to the wells and incubated for 1h at 37°C. Non-bound bacteria were removed by washing twice and bound bacteria were released and lysed by incubation with 1% SDS-0.1 M NaOH at 1h 60°C. Radioactivity was determined by liquid scintillation and the adhesion expressed as the percentage of radioactivity recovered after adhesion, relative to the radioactivity in the bacterial suspension added to the immobilized mucus.

7.2.1.3 Effect of pre-treatment with jejunal chyme on *Bifidobacterium* and LAB adhesion

In order to simulate digestion and study its effects on adhesion, bacteria were re-suspended in clear supernatant from jejunal chyme, a mixture of equal volumes from six dogs. After incubation for 1h at 37°C, the bacteria were washed in PBS and used in an adhesion assay as out-lined above (Kirjavainen et al 1998, Ouwehand et al 1999b).

7.2.1.4 Competitive exclusion assay (II)

Competitive exclusion of the canine pathogens by lactic acid producing bacteria was examined as described earlier (Ouwehand et al 2001a). Briefly, the mucus stocks were thawed and centrifuged to remove any precipitate formed during storage and diluted in

HH to a concentration of 0.5 mg ml⁻¹. The mucus was passively immobilized on polystyrene microtitre plate wells (Nunc Maxisorp, Roskilde, Denmark) by overnight incubation at 4°C. Excess mucus was removed by washing twice with HH. LAB and bifidobacteria without radiolabel were allowed to adhere to the immobilized mucus. Wells with PBS only served as control. Non-bound LAB and bifidobacteria were removed by washing twice with HH. Radio-labelled pathogenic bacteria were then added to the wells and incubated for 1h at 37°C. Non-bound pathogens were removed by washing twice and bound bacteria were released and lysed by incubation with 1% SDS-0.1 M NaOH for 1h at 60°C. Radioactivity of the lysed suspension was measured by liquid scintillation. The adhesion ratio (%) was calculated by comparing the radioactivities of the bacteria added and bound bacteria. Competitive exclusion was calculated as the percentage of pathogens bound after the initial adhesion of the lactic acid producing bacteria relative to pathogens bound in the absence of pre-adhered LAB or bifidobacteria (control).

7.2.1.5 Coaggregation (II)

Because it was observed that some LAB strains increase the adhesion of the canine pathogens, possible coaggregation of the LAB and the canine pathogens was investigated. The coaggregation test was performed as described earlier (Handley et al 1987). Bacterial suspensions were prepared as described above. The absorbance at 600 nm was adjusted to 0.5. Canine pathogens were mixed with an equal volume of an LAB strain and incubated for 4 h at 37°C. Absorbance at 600 nm was determined for the

mixture and for the bacterial suspensions alone. Coaggregation (%) was calculated according to the following equation:

$$\frac{OD_{600}(\text{pathogen alone} + \text{LAB alone})/2 - OD_{600}(\text{pathogen} + \text{LAB combination})}{OD_{600}(\text{pathogen alone} + \text{LAB alone})/2} \times 100\%$$

Where ‘pathogen alone’ and ‘LAB alone’ represent the optic density at 600 nm (OD_{600}) of the separate bacterial suspensions after 4h, and ‘pathogen + LAB combination’ represents the OD_{600} of the mixed bacterial suspension after 4h.

The rational for the method is that when bacteria coaggregate, they become large aggregates that are relatively dense and hence sediment, therefore ‘clearing’ the liquid phase giving a lower absorbance. The equation calculates the difference in sedimentation (as absorbance reduction) between the bacteria on their own and in combination.

7.2.2 Bacteria

7.2.2.1 Bacteria and their growth conditions used in adhesion assays

The lactic acid producing bacteria strains used and their culture conditions are listed in Table 2. The bacteria were grown from stocks stored at -75°C in 40% glycerol (1% inoculum). To metabolically radiolabel the bacteria, 10 μl of tritiated thymidine ([*methyl*-1,2- ^3H] thymidine, 120 Ci mmol^{-1}) was added to the medium (1 ml) . After

growth, the bacteria were harvested by centrifugation (2000 x *g*) and washed twice with phosphate buffered saline (PBS; pH 7.2; 10 mM phosphate) and resuspended in PBS. The absorbance was adjusted to 0.25 ± 0.02 in order to standardize the number of bacteria (10^7 - 10^8 CFU ml⁻¹) before use in an adhesion assay.

Strain		Origin/product	Growth medium	Atmosphere	Incubation time
<i>Lactobacillus rhamnosus</i>	GG	Gefilus® (Valio Ltd.)	MRS	Anaerobic	O/N
<i>Bifidobacterium lactis</i>	Bb12	(Chr. Hansen A/S)	MRS	Anaerobic	O/N
<i>Lactobacillus reuteri</i>	UK1A	dog faeces	MRS	Anaerobic	O/N
<i>Lactobacillus reuteri</i>	SK2A	dog jejunal chyme	MRS	Anaerobic	O/N
<i>Enterococcus faecium</i>	M74	Lactiferm® (Medipharma AB)	MRS	Anaerobic	O/N
<i>Enterococcus faecium</i>	SF273	Biobak® (Biofarm OY)	MRS	Anaerobic	O/N
<i>Lactobacillus johnsonii</i>	Lal	LC1 (Nestlé)	MRS	Anaerobic	O/N
<i>Lactobacillus casei</i>	Shirota	Yakult Singapore Pty., Ltd.	MRS	Anaerobic	O/N
<i>Lactobacillus delbrueckii</i> <i>subsp. bulgaricus</i>		ATCC 11842	MRS	Anaerobic	O/N

Table 2. Lactic acid producing bacteria strains used and their culture conditions

Abbreviations: MRS: de Man, Rogosa, Sharpe broth; O/N: over-night (12-14h)

7.2.2.2 The growth conditions of bacteria used in competitive exclusion assay

Pathogens used in the competitive exclusion assay were *C. perfringens*, *Salmonella enterica* Typhimurium, *C. jejuni* 517C/R and *Staphylococcus intermedius* EELA 2997₂/99; of canine origin. Their culture conditions are listed in Table 1, Study II.

To metabolically radiolabel the bacteria, 10 µl/ml tritiated thymidine ([*methyl*-1.2-³H-thymidine, 120 Ci mmol⁻¹) was added to the medium for competitive exclusion assay of the canine pathogens . Lactic acid producing bacteria were grown in the absence of tritiated thymidine. After growth, the bacteria were harvested by centrifugation (2000 x g), washed twice with PBS and re-suspended in PBS. The absorbance at 600 nm was adjusted to 0.5 ± 0.02 in order to standardize the number of bacteria (10^7 - 10^8 CFU ml⁻¹) before use in the competitive exclusion assay.

7.2.2.3 Bacteria used in coaggregation assay

For the coaggregation assay, the canine pathogens were grown under the same conditions as for the competitive adhesion assay, but without added tritiated thymidine.

7.2.3 IgA assays

7.2.3.1 Samples

For the duodenal brush samples, a prelubricated microcentrifuge tube (Costar® cat. no. 3207, Corning Inc. NY 14831, USA) was filled with 0.3 ml of Dulbecco's PBS (Sigma, St. Louis, MO, USA). The brush was washed in the PBS solution and the washing was stored at -70°C until assay.

Cotton swabs used to collect saliva were allowed to stand in the microcentrifuge tube filled with 0.3 ml Dulbecco's PBS for five minutes and were then squeezed against the tube wall to dry the swab. The dilution was stored at -70°C until assay.

7.2.3.2 IgA determinations

IgA concentrations in duodenal samples, saliva and sera were measured by an enzyme-linked immuno sorbent assay (ELISA). Microtiter plates (Maxisorp, Nunc Intermed, Denmark) were coated with polyclonal goat antibody to dog IgA (Nordic Immunological Laboratories, Drawer, CA, USA), 0,25 µg/well in 0.05 mol l⁻¹ Na₂CO₃ buffer (pH 9.2) and stored overnight at 4°C. After washing the wells, 100 µl of serial canine IgA standard dilutions from 5 to 0.05 mg l⁻¹ (IgA reference standard from the single radial immunodiffusion [SRID] kit by Veterinary Medical Research and Development Inc., Pullman, WA, USA) and samples (final dilutions 100-fold for duodenal samples and

saliva, and 1200-fold for sera) were added to the wells and incubated at room temperature for one hour. For all dilutions and washings PBS (0.05 mol l^{-1} phosphate, pH 7.3), containing 0.05% Tween 20 was used. The unbound material was removed with the washing buffer, and 100 μl of 400-fold diluted rabbit antibodies to canine IgA (Nordic Immunochemical Laboratories) was added and incubated at room temperature for one hour. After washings, 100 μl of 1000-fold diluted alkaline phosphatase conjugated goat antibody to rabbit IgG (Behring Mannheim, Indianapolis, IN, USA) was added and incubated at 37°C for 30 minutes. After washings, the amount of alkaline phosphatase fixed to the wells was determined in diethanolamine (1.0 mmol l^{-1})-magnesium chloride (0.5 mmol l^{-1}) buffer, pH 10.0 (Orion Diagnostica, Espoo, Finland), using p-nitrophenylphosphate (Sigma, St. Louis, MO, USA) as substrate for one hour at room temperature. The absorbance of the p-nitrophenolate liberated was measured at 405 nm with a 340 ATC microtitration plate reader (SLT, Labinstrument, Vienna, Austria). The intra- and interassay coefficients of variation of the assay were 6.1% and 7.9%, respectively, and the detection limit of the assay 0.1 mg/ml.

Protein concentrations of duodenal samples were determined using the Folin-Ciocalteu phenol reagent (Henry and Szustkiewics 1974). Human albumin (A-1653, Sigma, St. Louis, MO, USA) was used as standard.

7.3. Statistical analysis

The results from the adhesion experiments are expressed as the average of at least three independent experiments. Each experiment was performed with four parallels, to adjust for intra-experimental errors. A non-parametric Wilcoxon signed rank test was used to evaluate the statistical difference ($P < 0.05$) of competitive exclusion and coaggregation of each strain in comparison to the control. All statistical analysis was performed with StatView® (Abacus, Berkeley, USA).

The IgA concentrations of the duodenal samples were obtained as a mean of four different brush samples, each analysed in triplicate. Associations between different parameters were evaluated using the non-parametric Spearman rank correlation test. P-values of less than 0.05 were considered statistically significant.

8. RESULTS

8.1 Adherence to intestinal mucus

8.1.1 Ability of probiotic bacteria and canine indigenous LAB to adhere to canine intestinal mucus *in vitro*

The adhesion between tested strains was found to range from 0.5% (*L. casei* strain Shirota) to 35% (*L. rhamnosus* GG). *L. rhamnosus* GG was found to adhere significantly better than all other tested strains ($P<0.001$). *B. lactis* Bb12 adhered 9.4%, which is significantly different from all other tested strains ($P<0.005$), while *L. casei* strain Shirota adhered significantly less than all other tested strains ($P<0.05$). The adhesion percentages of all examined LAB are shown in Figure 1, Study I.

8.1.2 Effect of jejunal chyme treatment on lactic acid producing bacteria adhesion

Pre-treatment of the tested lactic acid producing bacteria with clear jejunal chyme significantly reduced the adhesion of all tested strains ($P<0.05$), with the exception of *L. johnsonii* La1 and *L. casei* strain Shirota. *L. rhamnosus* GG exhibited still the highest adhesion, but this was reduced to 7.8% and was not different from *B. lactis* Bb12, 4.8% adhesion. The adhesion of *L. casei* strain Shirota was, after pre-treatment with jejunal chyme, not different from the *Enterococcus* strains tested and the lactobacilli isolated

from dogs ($P>0.05$). The results of the jejunal chyme treatment are also expressed in Figure 1, Study I.

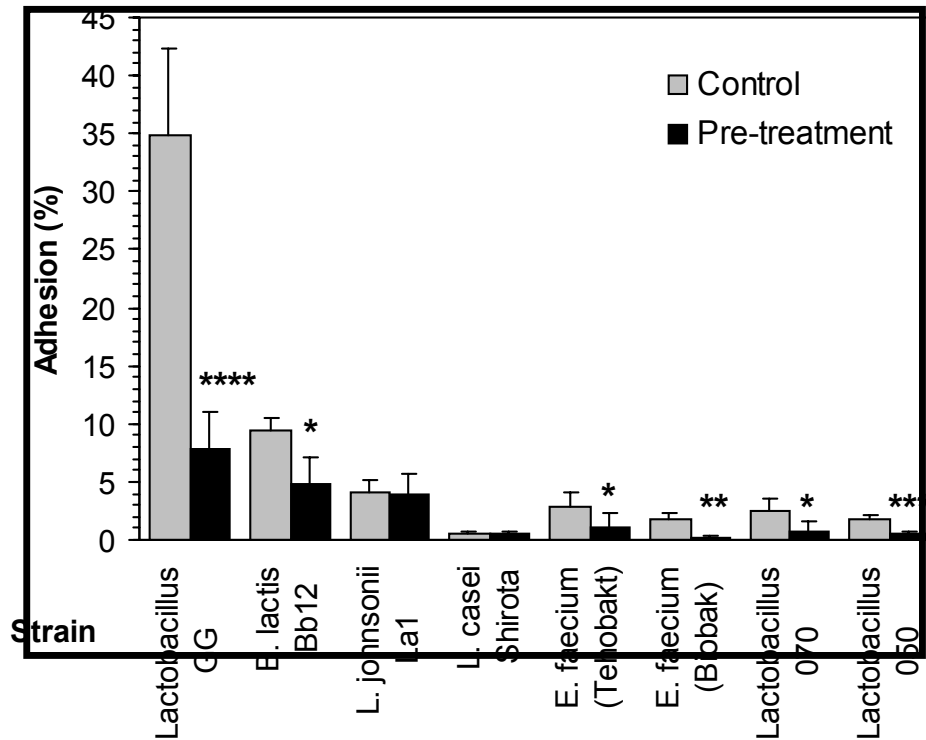


Figure 1. Effects of jejunal chyme treatment on *in vitro* adhesion of lactic acid producing bacteria

8.2 Ability of lactic acid producing bacteria to interfere with pathogen mucus adhesion *in vitro*

All the pathogens tested showed poor to moderate (1.2% to 5.1%) adhesion to the canine immobilized intestinal mucus. The average adhesions were as follows: *S. intermedius*

4.5% (2.74% to 5.6%); *S. enterica* Typhimurium 1.2% (1.1% to 1.4%); *C. jejuni* 5.1% (3.8% to 6.0%) and *C. perfringens* 3.0% (1.9% to 5.5%).

Adhesion of *C. perfringens* was reduced significantly by all tested LAB strains, between 53.7% and 79.1% of the control without lactic acid producing bacteria (Table 2, Study II). When compared with other lactic acid producing bacteria, the reduction was significantly lower with the strain *L. reuteri* UK1A, but not with *L. reuteri* SK2A. Both tested enterococci significantly enhanced the adhesion of *C. jejuni*, to 134.6% and 205.5% of the control without LAB, $p < 0.05$ (Table 2, Study II). The adhesion of the other tested pathogens was not significantly affected by the lactic acid producing bacteria.

8.3 Bacterial coaggregation

Significant coaggregation ($p < 0.05$) was observed for *L. rhamnosus* GG, *B. lactis* Bb12 and *L. reuteri* UK1A with *C. jejuni*, (Table 3, Study II). *L. reuteri* UK1A also exhibited coaggregation with *S. intermedius* (Table 3, Study II). No other significant coaggregation was observed. Coaggregation was not related to the enhanced *C. jejuni* adhesion caused by *E. faecium* strains (Table 3, Study II).

8.4 Host specificity of *in vitro* mucus adhesion

Adhesion to intestinal mucus of the tested strains was found to range from 1.06% (the average adhesion of *L. casei* Shirota to possum mucus) to 42.1% (the average adhesion of

L. rhamnosus GG to emu mucus). When compared to other lactic acid producing bacteria strains, *L. rhamnosus* GG was found to adhere significantly better ($P < 0.05$) to the intestinal mucus of all tested animal species (33.4% to 42.1%), with the exception of mucus from rainbow trout (17.6%). *L. casei* Shirota was found to have the poorest adherence of tested lactic acid producing bacteria to all animal species included in this study (1.1% to 1.8%). In human mucus the adhesion of *L. reuteri* UK1A was lowest. The results are expressed in Figure 2, which, for the sake of comparison, also shows a compilation of the results from earlier works by Tuomola et al (1999a) and Nikoskelainen et al (2001) . In Figure 2, the results from the work done by Tuomola and colleagues include adhesion studies with *L. rhamnosus* GG and *L. casei* Shirota. All the results from fish mucus adhesion are by Nikoskelainen and co-workers (2001) except for *E. faecium* SF68 and both *L. reuteri* strains. In general, no host specificity was observed in Study III, but there was a clear trend suggesting that the mucin adhesion of the tested lactic acid producing bacteria is dependent on the microorganism. (Figure 2).

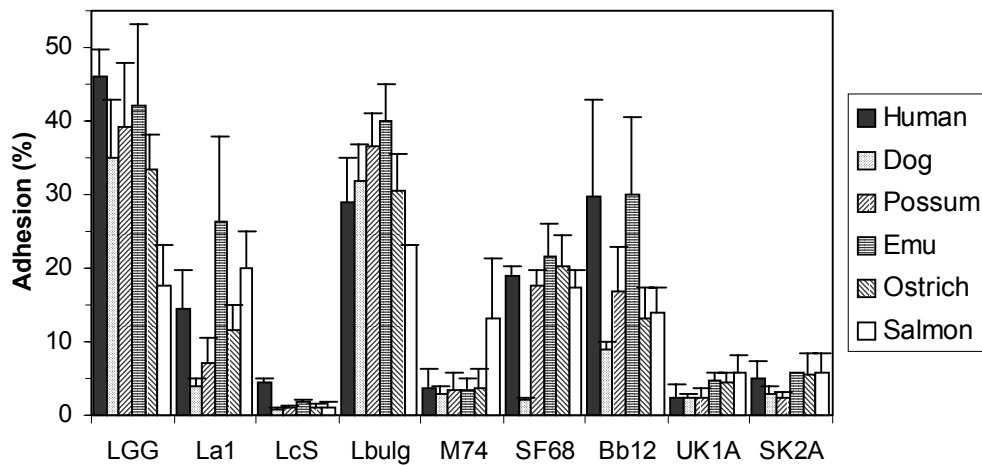


Figure 2. Comparison of lactic acid producing bacteria adhesion percentages within various hosts

Figure 2 comprises the results from Study III and studies by Tuomola et al (1999a) and Nikoskelainen et al (2001).

Abbreviations: LGG: *Lactobacillus rhamnosus* GG; La1: *Lactobacillus johnsonii* La1; LcS: *Lactobacillus casei* Shirota; Lbulg: *Lactobacillus bulgaricus*; M74: *Enterococcus faecium* M74; SF68: *Enterococcus faecium* SF68; Bb12: *Bifidobacterium lactis* Bb12; UK1A: *Lactobacillus reuteri* UK1A; SK2A: *Lactobacillus reuteri* SK2A

8.5 IgA concentrations and their reciprocal correlations

8.5.1. Serum IgA concentrations

IgA concentrations in the tested sera varied from 0.7 mg ml⁻¹ to 2.6 mg ml⁻¹ (mean 1.8 mg ml⁻¹ standard deviation, SD, 0.6 mg ml⁻¹) (Table 1, Study IV). These results are in line with the concentrations reported earlier (Heddle and Rowley 1975).

8.5.2 Duodenal IgA concentrations

In order to compensate for any disparity in duodenal sample dilutions, the IgA concentration of the sample was related to the concomitant concentration of total protein and the results are thus expressed as IgA/protein ratio. Ratios ranged from 0.009 to 0.153 (Table 1, Study IV). A significant negative correlation ($r=-0.64$, $p=0.0059$) was found between duodenal IgA/protein ratios and serum IgA concentrations. Samples from different parts of the duodenum of the same subject had different IgA/protein ratios, suggesting spatial variation (Table 1, Study IV).

8.5.3 Saliva IgA concentrations

Also the results from the saliva samples are expressed as IgA/protein and were found to range from 0.009 to 1.083 (Table 1, Study IV). The correlation between duodenal and

salivary IgA/protein ratio was not found to be significant ($p>0.05$). Nor was any correlation observed between the salivary and duodenal IgA/protein ratios ($p>0.05$).

9. DISCUSSION

9.1 Using the *in vitro* mucus adhesion model for adhesion studies in dogs

Adhesion to the intestinal mucosa is one of the main selection criteria for potential probiotic microorganisms (Ouwehand et al 1999a) and was therefore the subject of the current study. In the study, we compared the adhesion to immobilized canine intestinal mucus of probiotics intended for human use (4 strains) and animal use (2 strains) and two *L. reuteri* strains isolated from dogs. The strains for human use were chosen since they have well documented health effects (Salminen et al 1998a) and have been shown to be safe (Salminen et al 1998b).

The probiotics intended for human use were observed to bind to canine jejunal mucus in a way similar to the binding to human mucus observed earlier (Kirjavainen et al 1998, Tuomola et al 1999a). This suggests that the often mentioned species specificity of probiotics (Casas et al 1998) does not interfere with the *in vitro* adhesion of the tested strains. The probiotics intended for animal use, two *E. faecium* strains, exhibited a relatively low level of adhesion. Surprisingly, also both *L. reuteri* strains isolated from dogs showed a low level of adhesion. Based on the results obtained in this study, the intestinal mucus model provides a feasible method for the tentative evaluation of canine probiotics.

9.2 Effect of the canine jejunal chyme pre-treatment on probiotic and LAB adhesion

Exposure of the strains to jejunal chyme was observed to significantly reduce the adhesion of all tested strains, with the exception of *L. johnsonii* La1 and *L. casei* strain Shirota. It remains to be determined which of the components in jejunal chyme; enzymes, mucus, bile, etc. are responsible for the observed reduction in adhesion. However, it has earlier been observed that the treatment of *L. rhamnosus* GG with proteases reduces its adhesive abilities (Tuomola et al 2000), suggesting a possible role for proteolytic degradation in the observed effect. In addition, contact with digestive enzymes and bile affected the adhesion of selected probiotic LAB *in vitro* (Ouwehand et al 2001b). The observations also suggest that the adhesion observed *in vitro* may be quite different from *in vivo* after exposure to digestive juices. The selection criterion "adhesion" for probiotics should therefore perhaps be modified to "adhesion after exposure to digestive juices". It also indicates that the adhesive properties of probiotics should be tested after passage through an intestinal model or an animal model. The jejunal chyme can in future also be used for simulated passage through the small intestine in order to assess, *in vitro*, the survival of potential probiotics in this part of the gastrointestinal tract.

9.3 *In vitro* competitive exclusion of pathogenic bacteria by specific probiotic and indigenous canine LAB

The *in vitro* mucus adhesion of *C. perfringens* was significantly reduced by all tested LAB, which can be speculated to leading to a lower level of colonization of *C. perfringens*. the results from these studies could imply Reduced *C. perfringens* adhesion does not appear to be related to the adhesive abilities of the tested LAB. The mechanism for this reduction in pathogen adhesion remains therefore obscure, although it could imply a potential probiotic use of certain LAB strains in diminishing the number of *C. perfringens* organisms in the canine intestinal tract.

The adhesion of *S. Typhimurium* was not significantly affected by any of the tested LAB, in contrast to the findings of Tuomola and colleagues (1999b). In their study, two strongly adhesive strains of lactobacilli significantly increased *in vitro* adhesion of *S. Typhimurium* to the human intestinal mucus. In study I *L. Rhamnosus* GG was observed to have a very strong adhesion also to the canine intestinal mucus. This probably indicates that powerful adhesion itself does not enhance the binding of *S. Typhimurium* and that increased binding to the mucus is due rather to an unelucidated mechanism, which could be species specific. The adhesion of *S. intermedius* was not altered by any of the LAB included in this study.

Alarmingly, both *Enterococcus* strains were observed to significantly enhance the adhesion of *C. jejuni*. In addition to food and water borne infections, pets (particularly

young animals with diarrhoea) are considered to be a probable source of human *C. jejuni* infection (Ketley 1997). *Campylobacter* species have a typical corkscrew motility that enables them to penetrate through the mucus after they attach themselves to the mucosal surface (de Melo and Pechere 1988, Szymanski et al 1995, Sylvester et al 1996, Wooldridge and Ketley 1997). Adhesion to mucus receptors has been postulated to enhance this penetration by allowing the organism to attach on the top of the mucosal layer before travelling through the mucus to underlying intestinal epithelial cells (Sylvester et al 1996). *C. jejuni* lipopolysaccharide has been documented to act as an adhesin enabling the organism to adhere to mucin receptors, but binding to mucus varies among different strains (McSweeney and Walker 1986). Mucin fucose residues are considered chemotactic for *C. jejuni* (Hugdahl et al 1988). In Study II, the lack of adhesion enhancement with other pathogens could be the result of a different type of mucin adherence and could also be strain-dependent. The mechanism by which the enterococci enhance this first line adhesion of *C. jejuni* requires further investigation. Enhanced adhesion could be of clinical importance, as *E. faecium* supplementation has been reported to increase *Campylobacter* spp. counts in canine faeces (Vahjen and Männer 2003).

Acute intestinal disorders of dogs are often treated with probiotics and many of the commercial products contain *Enterococcus* species. Study II indicates that probiotic *E. faecium* may favour the adhesion and colonization of *C. jejuni* in a dog's intestine. In addition, also these *in vitro* findings have later been supported by a clinical study (Vahjen and Männer 2003), indicating that the *in vitro* adhesion model may be used as a tentative

method for evaluating the *in vivo* effects on pathogen colonization in the gut. The results suggest that *E. faecium* supplementation may make the dog a potential carrier of *C. jejuni* and a possible source for human campylobacteriosis. Enhanced colonization by this potential pathogen could thus be an additional risk factor for enterococci in feed or probiotic use. However, enterococci are widely distributed in nature and are also used in food technology, so not all the strains can be considered as a health risk. No probiotic enterococcal infections have been reported in veterinary medicine, so the risk, according to present knowledge, appears to be limited. However, research into probiotic therapy in companion animals has been scarce and it may be conceivable that possible enterococcal infections from probiotics have perhaps been overlooked. Furthermore, probiotics are often used on debilitated animals and in conjunction with antibiotics. Thus, the potential risk of promoting the growth of a zoonotic pathogen should be weighed against the positive health effects exerted by the probiotic. The *in vitro* adhesion model provides a feasible tool for a preliminary evaluation of potential alterations in bacterial adhesion caused by specific probiotic strains.

9.4 Coaggregation

Coaggregation may be one mode of communication between bacteria mediated by adhesins on one bacterial genus or species and equivalent receptors on another. Bacterial coaggregation is a well-known phenomenon in the oral cavity, where it is considered important for the formation of oral biofilm. This interaction can lead to rich growth, where the bacteria involved do not grow independently (Egland et al 2001). Some LAB coaggregated with *Escherichia coli* in the urogenital tract (Reid et al 1988). Intestinal lactobacilli have also been shown to coaggregate with enteropathogens; porcine lactobacilli coaggregated with *E. coli* K88 (Spencer and Chesson 1994). Coaggregation is thought to be beneficial if LAB produce antimicrobials since the ability of inhibitor-producing LAB to interact with a pathogen in close vicinity may be an important defence mechanism of normal flora (Reid et al 1988). In Study II, coaggregation was found to be an unlikely reason for enhanced binding with enterococci. However, it can be argued that the method used in Study II to measure coaggregation was perhaps not sensitive enough. The canine faecal strain *L. reuteri* UK1A coaggregated with *S. intermedius*, which is known to have the anal mucosa as a reservoir (Saijonmaa-Koulumies and Lloyd 1996). This interaction could be of ecological importance for colonization by this skin pathogen. Although statistically significant, the coaggregation in Study II was in general small, thus, its biological significance is uncertain.

9.5 Host specificity of *in vitro* *Bifidobacterium* and LAB adhesion to intestinal mucus

The lack of host-species specificity in bifidobacteria and LAB adhesion observed in Study III supports the conclusions from earlier studies (Kirjavainen et al 1998, Nikoskelainen et al 2001). This suggests mucus adhesion of certain lactic acid producing bacteria to be strain dependent rather than host dependent. This concurs with earlier reports of LAB adhesion in different animals (Barrow et al 1980, Mäyrä-Mäkinen et al 1983). Barrow and colleagues (1980) observed that some lactobacilli from pigs, wild boar and chickens adhered to pig (porcine?) squamous epithelial cells *in vitro*. They also noted that many of the LAB did not adhere to pig epithelial cells, even when the LAB were of porcine origin. Mäyrä-Mäkinen and colleagues (1983) found that adhesive *L. fermentum* strains isolated from calves also adhered to pig cells. It can be discussed whether the non-adhering strains would also have been non-adhering to their original host. This was something the above reports did not elucidate on. Hence the adhesion divergences noted in these reports could actually have been due to different, species- or strain-dependent adhesion factors of bifidobacteria and LAB involved in the study. However, the papers did not systematically investigate host specificity as was done in Study III.

The adhesive ability of *L. rhamnosus* GG was found to be superior to other bacteria examined in Study I. It has been reported to have excellent adhesion also to human mucus *in vitro* (Tuomola et al 1999a, Kirjavainen et al 1998) and in rainbow trout *in vitro* (Nikoskelainen et al 2001). Otherwise bifidobacteria and LAB adhesion percentages in rainbow trout and birds did not differ significantly from those observed in other species.

However, the aquatic environment of cold-water fish differs markedly from the living conditions of mammals and birds. Also the body temperature of birds is higher than that of mammals. In Study III, adhesion was examined only at 37 °C, which does not simulate the natural conditions of the intestines of rainbow trout or birds. The effect of incubation temperature on bifidobacteria or LAB adhesion in birds and fish remains to be determined and should be further explored.

Some bacteria have well-described, clearly species-specific adhesion mechanisms, such as *E. coli* K88 fimbriae specific to pigs (Jin and Zhao 2000). On the other hand, the ability to bind to and colonize intestinal mucosa across species boundaries is a well-known feature of many zoonotic pathogens: for example, the ubiquitous *S. enterica* serovars Typhimurium and Enteritidis can infect a wide range of hosts (Uzzau et al 2001). Many animal species are also known to serve as reservoirs for *C. jejuni* (Nachamkin 1997). Findings from Study III and the available literature suggest that also the adhesion trait of beneficial lactic acid producing bacteria may be more pronounced by the bacteria and intestinal microbiota rather than the host species itself. However, only *in vitro* adhesion has been evaluated in this study. *In vitro* adhesion cannot be considered as reliably predicting the *in vivo* colonization of intestinal microbiota because interspecies differences in intestinal physiology may have a marked effect on microbial colonization and persistence. Bacterial adhesion alone is not responsible for potential host specificity of intestinal microbiota.

Fuller (1973), Barrow and colleagues (1980) along with Mäyrä-Mäkinen and colleagues (1983) examined LAB adhesion to epithelial cells. Study III evaluated adhesion to intestinal mucus. As intestinal mucosa is the first contact surface for bacteria in the gut, the ability to adhere to mucus at a high level is of ecological importance to bacteria when colonizing the gut mucosa (Mikelsaar et al 1998). *In vitro* evaluation of LAB adhesion to intestinal mucus has been proved to be a suitable model for studying the probiotic adhesion (Kirjavainen et al 1998). Results from Study III imply that the attachment of the studied LAB and bifidobacteria to mucus is not determined by specific receptors in the host tissue. Although it is known, that the composition of mucus varies between animal species (including man), and also between different parts of the intestinal tract, human and canine mucus are known to share also similar characteristics (Forstner 1978, Verma and Davidson 1993). There may be some universal features in mucus common to different animal species (including man), enabling certain microbes, including LAB, to adhere to mucus better than others.

Species specificity is considered important for the temporary colonization required to initiate beneficial health effects, such as immunostimulation (Salminen et al 1998). Host specificity was challenged earlier by Conway and co-workers (1987). They reported similar adhesion of lactobacilli to porcine and human epithelial cells and concluded that the adhesion was non-specific, suggesting the pig intestinal cells could be used *in vitro* to screen the adhesion properties of LAB intended for human consumption. Whilst the results obtained in Study III support the use of animal models for probiotic studies, further studies are needed to investigate whether determinants other than mucus adhesion

are required to stimulate health effects. These results may also imply that probiotic strains isolated from humans may be beneficial for animal use, too (Nikoskelainen et al 2001b). This may have important safety implications: strains shown to be safe for humans can be fed to livestock and pets without a potential safety concern for the consumer or owner. It is for additional studies to investigate whether the highly binding LAB also initiate similar immune effects in animals as they do in humans.

In conclusion, *in vitro* mucus adhesion evaluation seems to be a promising model for tentative screening for the adhesive properties of potential canine probiotics. However, an *in vitro* model cannot truly represent the hostile conditions in the intestinal tract. To further validate this model for canine probiotic studies, complementary *in vivo* studies are needed.

9.6 Assessing duodenal sIgA concentration in dog

Results from Study IV report on a sensitive and reproducible sandwich ELISA for assessing duodenal and salivary sIgA and S-IgA. A significant negative correlation ($r = -0.64$, $p = 0.0059$) was found between duodenal sIgA/protein ratios and S-IgA concentrations. Saliva sIgA/protein ratios did not correlate with sIgA/protein ratios of duodenal samples.

In previous studies (Hedde and Rowley 1975, De Buysscher et al 1988), sIgA concentration has been determined from intestinal washes. These methods require

dissection of a considerable portion of intestine and are therefore not suitable for clinical use or serial measurements. Evaluation of intestinal sIgA production can be made by immunohistochemical documentation of IgA-containing cells in lamina propria. However, the IgA-impairment in German shepherds is believed to be due to defective synthesis or secretion of IgA, rather than an insufficient number of IgA-producing cells (Batt et al 1991). This renders the value of immunohistochemistry less applicable when assessing intestinal sIgA competence.

In contrast, brush samples are easily obtained as part of a routine endoscopic examination and give more accurate information about the intestinal immunological defence capability than S-IgA assessment or immunohistochemical measurement of IgA-producing plasma cells. Brush samples provide a tool for serial measurements as well.

A marked intra-individual variance was present in the intestinal IgA/protein ratios obtained in Study IV. An explanation for this may be that intestinal IgA has a patchy distribution instead of being an "even lining" on the gut mucosa. Also some of the IgA detected here could have originated from serum, and therefore not be sIgA. The lack of commercially available canine anti-secretory component hindered a more precise structural analysis of the IgA measured in Study IV. The observation of a possible uneven distribution of IgA on the intestinal mucosa warrants further investigation on its cause and biological role.

Results from Study IV add more confirmation to the conception that assessment of the IgA competence of dogs cannot be based on serum IgA concentration. Many studies

associate a very low S-IgA level (less than 0.20 mg ml^{-1}) with selective IgA deficiency and chronic gastrointestinal, respiratory and dermatological infections (Felsburg et al 1985, Campbell et al 1991, Guildford 1996). This may be due to impaired IgA production in plasma cells, thus resulting in low IgA concentrations both in serum and on mucosal surfaces.

Selective IgA deficiency is a common immunological disorder in dogs (Campbell et al 1991). However, many of these individuals are asymptomatic despite a low S-IgA level (Felsburg et al 1985, Campbell et al 1991, Ginel et al 1993, Guildford 1996). It can be hypothesized that in dogs with low S-IgA, the mucosal sIgA concentration may be sufficient to maintain a local immune response. The S-IgA concentrations of the dogs involved in Study IV were within normal limits. Therefore, it remains to be examined whether the dogs with low S-IgA (less than 0.20 mg ml^{-1}) concentrations do have compensatory high duodenal sIgA/protein levels.

The role of circulating S-IgA is not fully understood. In humans, IgA can be divided into two subclasses, which are distributed differently between the systemic and mucosal immune systems (Heremans 1974). It is not known whether such classification applies to canine IgA, too. If similar subclasses are present also in dogs, it can be hypothesized that sIgA is focused mainly on antimicrobial defense on the mucosal surface and that S-IgA acts primarily as a regulator of immunity. Although both canine sIgA and S-IgA originate mostly from gut-associated lymphoid tissue (Heremans 1974), they may be regulated independently and therefore there is no correlation between sIgA and S-IgA

concentrations. Mestecky and colleagues (1999) suggested that circulating S-IgA has an important role in preventing an immune response-inflammation cycle, which can result in tissue damage and chronic inflammatory disease.

Based on the significant negative correlation between sIgA and S-IgA found in Study IV, it can be postulated that S-IgA is needed to eliminate hostile invaders from the circulatory system and body tissues if sIgA fails and intruding antigens are able to penetrate the epithelium. In this case, S-IgA is preferred to IgG and IgM because of its milder response, which is less likely to cause damage to the host's tissue (Willard 1992, Mestecky et al 1999).

Study IV found no correlation between duodenal and saliva sIgA levels, rendering saliva sIgA assays dubious for assessing mucosal sIgA concentration in the gut. Certain animal models have shown that mucosal immunization leads also to serum IgA response and may induce mucosal IgA responses in distant mucosal surfaces, too (Bergmeier et al 1995, Wu and Russel 1998, Jakobsen et al 2002). However, there has been divergence in the results: the responses are not always comparable between different mucosal surfaces and serum (Currie et al 2001, Heddle and Rowley 1975).

It has been suggested that the majority of sIgA found on mucosal surfaces emerges from local synthesis rather than from the transport of S-IgA from the blood, stressing the importance of local sIgA production (Mestecky 1993). This could explain the lack of

correlation between duodenal and saliva sIgA concentrations observed in Study IV and further supports the need for an easy method for assessing intestinal sIgA production.

Large day-to-day and diurnal variations in saliva IgA have been reported (Ginel et al 1993). Whether this fluctuation also applies to the intestinal tract needs to be determined as it may influence the interpretation of study results in the future. The method described in Study IV to sample the intestinal mucosa is gentle and allows for the examination of temporal variation.

10. CONCLUSIONS

I Studies on the adhesive properties of probiotic and indigenous canine LAB in intestinal mucus model *in vitro* led to following conclusions:

1. Intestinal mucus model utilized in human probiotic studies is applicable also when examining the adhesive properties of potential probiotic LAB intended for canine use. This *in vitro* adhesion assay may provide a basis for studying the efficacy of these probiotics in dogs. Canine jejunal chyme is a convenient source of intestinal mucus. Some probiotics intended for human use were found to interact well with immobilized canine intestinal mucus.
2. Exposure of the strains to jejunal chyme was observed to significantly reduce the adhesion of all tested strains, with the exception of *L. johnsonii* La1 and *L. casei* strain Shirota. Therefore, adhesion observed *in vitro* may be quite different from that in *in vivo* after exposure to digestive juices. It remains to be determined which of the components in jejunal chyme; enzymes, mucus, bile, etc. are responsible for the observed reduction in adhesion. Canine jejunal chyme is a convenient source of intestinal mucus and can be used to assess the effects of digestion on probiotics *in vitro*.
3. The canine mucus adhesion model provides a tool for the preliminary evaluation of competitive exclusion of canine intestinal pathogens *in vitro*. The LAB tested

may be beneficial in reducing the number of *C. perfringens* organisms in the canine intestine. These LAB should be evaluated further for the treatment and/or prevention of disease *in vivo*. The observed increase in *in vitro* mucus adhesion of *C. jejuni* caused by the tested enterococci may be a cause for concern. Enhanced *C. jejuni* adhesion is a new potential risk factor of *E. faecium* and requires further investigation. This finding emphasizes the importance of establishing safety guidelines for probiotics intended for both animal and human use. The *in vitro* mucus adhesion model provides a feasible method for the tentative evaluation of potential canine probiotics.

4. Mucus adhesion properties are more dependent on the LAB strain than on the host animal, thus suggesting that mucus adhesion mechanisms may share some universal features common to mammals, birds and fish. This may suggest that the animal models in probiotic adhesion assays may be more reliable than previously thought. The documented positive health effects facilitated by adherent probiotics in humans may also denote the possibility of similar outcomes in other species and *vice versa*.

II The study to develop a method for intestinal sIgA assay yielded a novel method for repeated sampling of the duodenal mucosa to directly determine the intestinal immune response. This method may be utilized in further studies when examining the IgA-modulating properties of well-adhering, potential probiotics aimed for canine use. A marked variance in sIgA concentrations was measured from different sites of duodenal

mucosa. In addition, it can be concluded, that neither S-IgA nor salivary sIgA are suitable parameters for assessing duodenal sIgA competence in the dog.

Based on the findings of the study, it is clear that further studies are needed to evaluate the potential beneficial health effects of lactic acid producing bacteria intended for dogs. Certain probiotics may be useful in competitive exclusion of pathogenic *C. perfringens*. *In vivo* studies would benefit in validating the *in vitro* methods.

The immunomodulatory effects documented in humans are certainly of special interest also in veterinary science. This may be the most important field of probiotic science in future studies on dogs.

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12. REFERENCES

Adlerberth, I., Cerquetti, M., Poilane, I., Wold, A., Collignon, A. Mechanisms of colonisation and colonisation resistance of the digestive tract. *Microb. Ecol. Health Dis.* 2000; 11: 223-239.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., von Wright, A. Persistence of colonisation of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption. *Appl. Environ. Microbiol.* 1999; 65: 351-354.

Alvarez-Olmos, M.I., Oberhelman, R.A. Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. *Clin. Inf. Dis.* 2001; 32: 1567-1576.

Anon. Suomen Eläinlääkkeet. Helsinki, 2002: 76-99.

An, Y.H., Dickinson, R.B., Doyle, R.J. Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections. *In*: An, Y.H. and Friedman, R.J. (eds) *Handbook of bacterial adhesion: Principles, Methods and Applications*. Humana Press. Totowa, 2000: 1-28.

Axelsson, L. Lactic acid bacteria: classification and physiology. *In*: Salminen, S. and von Wright, A. (eds), Lactic acid bacteria: Microbiology and functional aspects. Marcel Dekker, New York, 1998: 369-383.

Barrow, P.A., Brooker, B.E., Fuller, R., Newport, M.J. The attachment of bacteria to the gastric epithelium of the pig and its importance in the microecology of the intestine. *J. Appl. Bacteriol.* 1980; 48: 147-154.

Barrows, G.T., Deam, B.D. using probiotics in small animals: A new approach. *Vet. Med.* 1985; 80: 36, 41-42.

Batt, R.M., Barnes, A., Rutgers, H.C., Carter, S.D. Relative IgA deficiency and small intestinal bacterial overgrowth in German shepherd dogs. *Res.Vet. Sci.* 1991; 50: 106-111.

Benno, Y., Mitsuoka, T. Development of intestinal microflora in humans and animals. *Bifidobact. Microflora.* 1986; 5: 13-25.

Benno, Y., Nakao, H., Uchida, K., Mitsuoka, T. Individual and seasonal variations in the composition of fecal microflora of beagle dogs. *Bifidobact. Microfl.* 1992; 11: 69-76.

Benyacoub, J., Czarnecki-Maulden, G.L., Cavadini, C., Sauthier, T., Anderson, R.E, Schiffrin, E.J., von der Weid, T. Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. J. Nutr. 2003; 133: 1158-1162.

Berg, R.D. The indigenous gastrointestinal microflora. Trends. Microbiol. 1996; 4: 430-435:

Bergmeier, L.A., Tao, L., Gearing, A.J., Adams, S., Lehner, T. Induction of IgA and IgG antibodies in vaginal fluid, serum and saliva following immunization of genital and gut associated lymphoid tissue. Adv. Exp. Med. Biol. 1995; 371B: 1567-1573.

Biourge, V., Vallet, C., Levesque, A., Sergheraert, R., Chevalier, S., Robertson, J. -L. The use of probiotics in the diet of dogs. J. Nutr. 1998; 128: 2730S-2732S.

Blaser, M.J., Musser, J.M. Bacterial polymorphisms and disease in humans. J. Clin. Invest. 2001; 107: 391-392.

Blomberg, L., Henriksson A., Conway, P.L. Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. Appl. Environ. Microbiol. 1993; 59: 34-39.

Buisine, M.P., Desreumaux, P., Leteurtre, E., Copin, M.C., Colombel, J.F., Porchet, N.,

Aubert, J.P. Mucin gene expression in intestinal epithelial cells in Crohn's disease. *Gut*. 2001; 49: 544-551.

Casas, I.A., Edens, F.W., Dobrogosz, W.J. *Lactobacillus reuteri*: an effective probiotic for poultry and other animals. *In*: Salminen, S. and von Wright, A. (eds), Lactic acid bacteria: Microbiology and functional aspects. Marcel Dekker, New York, 1998: 475-518.

Campbell, K.L., Neitzel, C., Zuckermann, F.A. Immunoglobulin A deficiency in the dog. *Canine Pract.* 1991; 16: 7-11.

Chou, L.-S., Weimer, B. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci.* 1999; 82: 23-31.

Clapper, W.E. Microbiology. Gastrointestinal tract. *In*: Anderson, A.C. and Good, L.S. (eds), The beagle as an experimental dog. Iowa State University, Ames, Iowa, 1970: 469-473

Conway, P.L., Gorbach, S.L. Goldin, B.R. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci.* 1987; 70: 1-12.

Cremonini F, Di Caro S, Santarelli L, Gabrielli M, Candelli M, Nista EC, Lupascu A, Gasbarrini G, Gasbarrini A. Probiotics in antibiotic-associated diarrhoea. *Dig. Liver Dis.* 2002; 34: S78-80.

Currie, C.G., McCallum, K., Poxton, I.R. Mucosal and systemic antibody responses to the lipopolysaccharide of *Escherichia coli* O157 in health and disease. *J. Med. Microbiol.* 2001; 50: 345-354.

Davis, C.P., Cleven, D., Balish, E., Yale, C.E. Bacterial association in the gastrointestinal tract of beagle dogs. *Appl. Environ. Microbiol.* 1977; 34: 194-206.

De Buysscher, E.V., Breitschwerdt, E.B., MacLachlan, N.J. Elevated serum IgA associated with immunoproliferative enteropathy of Basenji dogs: Lack of evidence for alpha heavy-chain disease or enhanced intestinal IgA secretion. *Vet. Immunol. Immunopathol.* 1988; 20: 41-52.

De Melo, M.A., Pechere, J.C. Effect of mucin on *Campylobacter jejuni* association and invasion of Hep-2 cells. *Microb. Pathog.* 1988; 5: 71-76.

Deplancke, B., Gaskins, H.R. Microbial modulation of innate defence: goblet cells and the intestinal mucus layer. *Am. J. Clin. Nutr.* 2001; 73: 1131S-1141S.

De Waard R., Snel J, Bokken G.C., Tan P.S., Schut F., Huis In't Veld J.H. Comparison of faecal *Lactobacillus* populations in experimental animals from different breeding facilities and possible consequences for probiotic studies. *Lett. Appl. Microbiol.* 2002; 34: 105-109.

Dieleman LA, Goerres MS, Arends A, Sprengers D, Torrice C, Hoentjen F, Grenther WB, Sartor RB. *Lactobacillus* GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. *Gut*. 2003; 52: 370-376.

Donohue, D.C., Salminen, S., Marteau, P. Safety of probiotic bacteria. *In*: Salminen, S., von Wright, A. (eds), Lactic acid bacteria. Microbiology and functional aspects. 2nd ed. Marcel Dekker Inc. New York, 1998: 369-383.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G.C., Shanahan, F., Collins, J., K. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am. J. Clin. Nutr.* 2001; 73: 386S-392S.

Edmond M.B. Wallace S.E. McClish D.K. Pfaller M.A. Jones R.N. Wenzel R.P.
Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin. Inf. Dis.* 1999; 29: 239-244

Edwards, R.A., Puente J.L. Fimbrial expression in enteric bacteria: a critical step in intestinal pathogenesis. *Trends Microbiol.* 1998; 6: 282-287.

Egland, P.G, Dû, L.D, Kolenbrander, P.E. Identification of independent *Streptococcus gordonii* SspA and SspB functions in coaggregation with *Actinomyces naeslundii*. *Infect. Immun.* 2001; 69: 7512-7516.

Elliott, S.E., Buret, A., McKnight, W., Miller, M.J.S., Wallace, J.L. Bacteria rapidly colonize and modulate healing of gastric ulcers in rats. *Am. J. Physiol.* 1998; 275: G425-G432.

Erickson, K.L., Hubbard, N.E. Probiotic immunomodulation in health and disease. *J Nutr.* 2000; 130: 403S-409S.

Felsburg, P.J., Glickman, L.T., Jezyk, P.F. Selective IgA deficiency in the dog. *Clin. Immunol. Immunopathol.* 1985; 36: 297-305.

Ferguson A, Humphreys, K.A., Croft N.M. Technical report: results of immunological tests on faecal extracts are likely to be extremely misleading. *Clin. Exp. Immunol.* 1995; 99: 70-75.

Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E. Enterococci at the crossroads of food safety. *Int. J. Food Microbiol.* 1999; 47: 1-24.

Fuller, R. Ecological studies on the *Lactobacillus* flora associated with the crop epithelium of the fowl. *J. Appl. Bacteriol.* 1973; 36: 131-139.

Fuller, R. Probiotics in man and animals. *J. Appl. Bacteriol.* 1989; 66: 365-378.

Genovese K.J., Anderson R.C., Harvey R.B., Nisbet D.J. Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic *Escherichia coli* infection in nursery-raised neonatal pigs. Can. J. Vet. Res. 2000; 64: 204-207.

German, A.J., Hall, E.J., Day, M.J. Measurement of IgG, IgM and IgA concentrations in canine serum, saliva, tears and bile. Vet. Immunol. Immunopathol. 1998; 64: 107-121.

Ginel, P.J., Novales, M., Lozano, M.D., Molleda, J.M., Lopez, R. Local secretory IgA in dogs with low systemic IgA levels. Vet. Rec. 1993; 132: 321-323.

Goldblum, R.M. The role of IgA in local immune protection. J. Clin. Immunol. 1990; 10: 64S-71S.

Gorbach, S.L. Probiotics and gastrointestinal health. Am. J. Gastroenterol. 2000; 1: S2-4.

Greetham, H.L., Giffard, C., Hutson, R.A., Collins. M.D., Gibson, G.R. Bacteriology of the Labrador dog gut: a cultural and genotypic approach. J. Appl. Microbiol. 2002; 93: 640-646.

Guandalini, S., Pensabene, L., Zikri, M.A., Dias, J.A., Casali, L.G., Hoekstra, H., Kolacek, S., Massar, K., Micetic-Turk, D., Papadopoulou, A., de Sousa, J.S., Sandhu, B., Szajewska, H., Weizman, Z. *Lactobacillus* GG administered in oral rehydration solution

to children with acute diarrhea: a multicenter European trial. J. Pediatr.Gastroenterol. Nutr. 2000; 30: 54-60.

Guarner, F., Malagelada, J.R. Gut flora in health and disease. Lancet. 2003; 8: 512-519.

Guildford, W.G. Gastrointestinal immune system. *In*: Guildford, W.G., Center, S.A., Strombeck, D.R., Williams, D.A., and Meyer, D.J. (eds.), Strombeck's Small Animal Gastroenterology 3rd ed, W.B.Saunders Company, Philadelphia, 1996: 20-39.

Haller D., Colbus H., Ganzle M.G., Scherenbacher .P, Bode C., Hammes W.P. Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: a comparative in vitro study between bacteria of intestinal and fermented food origin. Syst. Appl. Microbiol. 2001; 24: 218-226.

Handley, P.S., Hartey, D.W.S., Wyatt, J.E., Brown, C.R., Doran, J.P., Gibbs, A.C.C. A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of *Streptococcus salivarius*. J. Gen. Microbiol. 1987; 133: 3207-3217.

Haneberg, B., Kendall, D., Amerongen, H.M. Apter, F.M., Kraehenbuhl, J.-P., Neutra, M.R. Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina measured by a new method for collection of secretions from local mucosal surfaces. Infect. Immun. 1994; 62: 15-23.

Harmoinen, J., Mättö, J., Rinkinen, M., Wilsson-Rahmberg, M. Westermarck, E. Permanent jejunal fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function. *Comp. Med.* 2001; 51: 252-256.

Havenaar, R., Huis in't Veld, J.H.J. Probiotics: a general view. *In*: Wood, B.J.B. (ed) The lactic acid bacteria in health and disease. The lactic acid bacteria, Vol. 1. Chapman and Hall, New York, 1992: 209-224.

Hedde, R.J., Rowley, D. Dog immunoglobulins I. Immunochemical characterization of dog serum, parotid saliva, colostrum, milk and small bowel fluid. *Immunology*.1975; 29: 185-195.

Henry, R.J., Szustkiewics, C.P. Determination of protein in cerebrospinal fluid by the Folin-Lowry method. *In*: Henry, R.J., Cannon, D.C, and Winkelman, J.W. (eds), *Clinical Chemistry. Principles and techniques*. Hagerstown (Md.) Harper & Row. New York, 1974.

Heremans, J.F. The IgA system in connection with local and systemic immunity. *In*: Mestecky, J., and Lawton, A.R. (eds), *Advances in Experimental Medicine and Biology*, vol. 45. Plenum Press, New York, 1974: 3-11.

Hirano J., Yoshida T., Sugiyama T., Koide N., Mori I., Yokochi T. The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells *in vitro*. Microbiol Immunol. 2003; 47: 405-409.

Hirn J., Nurmi E., Johansson T., Nuotio L. Long-term experience with competitive exclusion and salmonellas in Finland. Int. J. Food Microbiol. 1992; 15: 281-285.

Hugdahl, M.B., Beery, J.T, Doyle, M.P. Chemotactic behaviour of *Campylobacter jejuni*. Infect. Immun. 1988; 56: 1560-1566.

Isberg R.R., Barnes, P. Dancing with the host: flow-dependent bacterial adhesion. Cell. 2002; 110: 1-4

Isolauri, E., Sütas, Y., Kankaanpää, P., Arvilommi, H., Salminen, S. Probiotics: effects on immunity. Am. J. Clin. Nutr. 2001; 73: 444S-450S.

Jakobsen, H., Bjarnarson, S., del Giudice, G., Moreau, M., Siegrost, C.-A., Jonsdottir, I. Intranasal immunization with pneumococcal conjugate vaccines with LT-K63, a nontoxic mutant of heat-labile enterotoxin, as adjuvant rapidly induces protective immunity against lethal pneumococcal infections in neonatal mice. Infect. Immun. 2002; 70: 1443-1452.

Jett, B.D., Huycke, M.M., Gilmore, M.S. Virulence of enterococci. Clin. Microbiol. Rev. 1994; 7: 462-478.

Jin, L.Z., Marquardt, R.R., Zhao, X. A strain of *Enterococcus faecium* (18C23) inhibits adhesion of enterotoxigenic *Escherichia coli* K88 to porcine small intestine mucus. Appl. Environ. Microbiol. 2000; 66: 4200-4204.

Jin, L.Z., Zhao, X. Intestinal receptors for adhesive fimbriae of enterotoxigenic *Escherichia coli* (ETEC) K88 in swine – review. Appl. Microbiol. Biotechnol. 2000; 5: 311-318.

Johnston, K.L. Small intestinal bacterial overgrowth. Vet. Clin. North Am. Small Anim. Pract. 1999; 29: 523-550.

Juntunen M, Kirjavainen PV, Ouwehand AC, Salminen SJ, Isolauri E. Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. Clin Diagn Lab Immunol. 2001; 8: 293-296.

Kanasugi, H., Hasegawa, T., Goto, Y., Ohtsuka, H., Makimura, S. Yamamoto, T. Single administration of enterococcal preparation (FK-23) augments non-specific immune responses in healthy dogs. Int. J. Immunopharmacol. 1997; 19: 655-659.

Ketley, J.M. Pathogenesis of enteric infection by *Campylobacter*. Microbiology. 1997; 143: 5-21.

Kirjavainen, P.V., Ouwehand, A.C., Isolauri, E., Salminen, S.J. The ability of probiotic bacteria to bind to human intestinal mucus. FEMS Microbiol. Lett. 1998; 167: 185-189.

Kruth, S.A., Prescott, J.F., Welch, M.K., Brodsky, M.H. Nosocomial diarrhea associated with enterotoxigenic *Clostridium perfringens* infection in dogs. JAVMA. 1989; 195: 331-334.

Lahrssen, M., Zentek, J. Efficacy of probiotic feed additives: guidelines for the evaluation of the efficiency of microorganisms in dogs, cats and horses. Dtsch. Tierärztl. Wschr. 2002; 109: 22-25.

Macfarlane, G.T., Cummings, J.H. Probiotics, infection and immunity. Curr. Opin. Infect. Dis. 2002; 15: 501-506.

Matsumura A, Saito T, Arakuni M, Kitazawa H, Kawai Y, Itoh T. New binding assay and preparative trial of cell-surface lectin from *Lactobacillus acidophilus* group lactic acid bacteria. J. Dairy Sci. 1999; 82: 2525-2529

McGroarty, J.A. Cell surface appendages of lactobacilli. FEMS Microbiol. Lett. 1994; 124: 405-410

McSweeney, E., Walker, R.I. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. Infect. Immun. 1986; 53: 141-148.

Metchnikoff, E. The prolongation of life. Optimistic studies. Butterworth-Heinemann. London, 1907.

Mestecky, J. Saliva as a manifestation of the common mucosal immune system. *Ann. New York Acad. Sci.* 1993; 694: 184-194.

Mestecky, J., Russel, M.W., Elson C.O. Intestinal IgA: Novel views on its function in the defence of the largest mucosal surface. *Gut.* 1999; 44: 2-5.

Mikelsaar, M., Mändar, R., Sepp, E. Lactic acid microflora in the human microbial ecosystem and its development. *In: Salminen, S., von Wright, A., (eds) Lactic acid bacteria. Microbiology and functional aspects.* 2nd ed. Marcel Dekker Inc. New York, 1998: 211-253.

Miller, R.S., Hoskins, L.C. Mucus degradation in human colon ecosystems. Faecal population densities of mucus-degrading bacteria estimated by a “most probable number” method. *Gastroenterology.* 1981; 81: 759-765.

Morata de Ambrosini V, Gonzalez S, de Ruiz Holgado AP, Oliver G. Study of the morphology of the cell walls of some strains of lactic acid bacteria and related species. *J Food Prot.* 1998; 61: 557-562.

Mouricout M. Interactions between the enteric pathogen and the host. An assortment of

bacterial lectins and a set of glycoconjugate receptors. *Adv Exp Med Biol.* 1997; 412: 109-123.

Mukai T, Asasaka T, Sato E, Mori K, Matsumoto M, Ohori H. Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. *FEMS Immunol Med Microbiol.* 2002; 32: 105-110.

Mäyrä-Mäkinen, A. Manninen, M, Gyllenberg, H. The adherence of lactic acid bacteria to the columnar epithelial cells of pigs and calves. *J. Appl. Bacteriol.* 1983; 55: 241-245.

Nachamkin, I. *Campylobacter jejuni*. *In*: Doyle, M.P., Beuchat, L.R., Montville, T.J. (eds), Food microbiology. Fundamentals and frontiers. ASM Press, Washington D.C., 1997: 159-170.

Neutra, M.R., Forstner, J.F. Gastrointestinal mucus: synthesis, secretion and function. *In*: Johnson, L.R., (ed), Physiology of the gastrointestinal tract. Vol. 2. 2nd Ed. Raven Press, New York, 1987: 975-1010.

Nikoskelainen, S., Salminen, S., Bylund, G., Ouwehand, A.C. Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. *Appl. Environm. Microb.* 2001a; 67: 2430-2435.

Nikoskelainen, S., Ouwehand, A.C., Salminen, S., Bylund, G. Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. Aquaculture. 2001b; 198: 229-236.

Nikoskelainen, S., Ouwehand, A.C., Bylund, G., Salminen, S., Lilius, E.M. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). Fish Shellfish Immunol. 2003; 15: 443-452.

Niv, Y., Turani, H., Fraser, G.M. Histochemical characterization of mucosal mucin in serial biopsies along the human colon. Digestion. 1996; 57: 109-112.

O'Halloran, F. M., Morrissey, S.D., Murphy, L., Thornton, G., Shanahan, F., O'Sullivan, G.C., Collins, J.K. Adhesion of potential probiotic bacteria to human epithelial cell lines. Int. Dairy J. 1998; 8: 596.

Ouwehand, A.C, Kirjavainen, P.V., Grönlund, M.-M., Isolauri, E., Salminen, S. Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J. 1999; 9: 623-630.

Ouwehand, A.C., Tuomola, E.M., Tölkkö, S., Salminen, S. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. Int. J. Food Microbiol. 2001a; 64: 119-126.

Ouwehand, A.C., Tölkö, S., Salminen, S. The effect of digestive enzymes on the adhesion of probiotic bacteria in vitro. J Food Sci. 2001b; 66: 856-859.

Ouwehand, A.C, Vesterlund, S. Health aspects of probiotics. IDrugs. 2003; 6: 573-580.

Parker, R.B. Probiotics, the other half of the antibiotic story. Anim. Nutr. Health, 1974; 29: 4-8.

Perdigon, G., Maldonado Galdeano, C., Valdez, J.C., Medici, M. Interaction of lactic acid bacteria with the gut immune system. Eur. J. Clin. Nutr. 2002; 56 Suppl 4:S21-26.

Pitman, R.S., Blumberg, R.S. First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system. J. Gastroenterol. 2000; 35: 805-814.

Reid, G., Burton, J. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microbes Infect. 2002; 4: 319-324.

Reid, G., McGroarty, J.A., Angotti, R., Cook, R.L. *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Can. J. Microbiol. 1988; 34: 344-351.

Pasupathy K., Sahoo A., Pathak N.N. Effect of *Lactobacillus* supplementation on growth and nutrient utilization in mongrel pups. Arch. Tierernahr. 2001; 55: 243-253.

Rolfe, R.D. The role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* 2000; 130: 396S-402S.

Saarela, M., Mogensen, G., Fondén, R., Mättö, J., Mattila-Sandholm, T. Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.* 2000; 84: 197-215.

Saijonmaa-Koulumies, L., Lloyd, D. Colonization of the canine skin with bacteria. *Vet. Dermatol.* 1996; 7: 153-162.

Salminen S., Bouley M.C., Boutron-Rualt M.C., Cummings J., Franck A., Gibson G., Isolauri E., Moreau M.-C., Roberfroid M., Rowland I. Functional food science and gastrointestinal physiology and function. *Br. J. Nutr.* 1998; 80: S147-S171.

Salminen, S., Deighton, M.A., Benno, Y., Gorbach, S.L. Lactic acid bacteria in health and disease. *In: Salminen, S., von Wright, A., (eds), Lactic acid bacteria. Microbiology and functional aspects.* 2nd ed. Marcel Dekker Inc. New York, 1998: 211 – 253.

Salminen, S., Ouwehand, A., Benno, Y., Lee, Y.K. Probiotics: how should they be defined? *Trends Foods Sci. Technol.* 1999; 10: 107-110.

Sanders, M.E. Probiotics: considerations for human health. *Nutr. Rev.* 2003; 61: 91-99.

Savage, D.C. Microbial ecology of the gastrointestinal tract. *Annu. Rev. Microbiol.* 1977; 31:107-133.

Schell, M.A., Karmirantzou ,M., Snel, B., Vilanova, D., Berger, B., Pessi, G., et al. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc. Natl. Acad. Sci.* 2002; 99: 14422-14427.

Schiffirin, E.J. Blum, S. Interactions between the microbiota and the intestinal mucosa. *European Journal of Clinical Nutrition*, 2002; 56: S60-S64.

Schiffirin, E.J., Brassart, D., Servin, A.L., Rochat, F., Donnet-Hughes, A. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. *Am. J. Clin. Nutr.* 1997; 66: 515S.

Schummer, A., Nickel, R. The alimentary canal, general and comparative. Intestines. *In*: Schummer, A., Nickel, R., (eds), *Textbook of the anatomy of the domestic animals*. Vol. II. The viscera of the domestic mammals. 2nd revised edition. Verlag Paul Parey, Berlin and Hamburg, 1979: 107-108

Shiba, T., Aiba, Y., Ishikawa, H., Ushiyama, A., Takagi, A., Mine, T., Koga, Y. The suppressive effect of bifidobacteria on *Bacteroides vulgatus*, a putative pathogenic microbe in inflammatory bowel disease. *Microbiol Immunol.* 2003; 47: 371-378.

Smith, H.W. Observations on the flora of the alimentary tract of animals and factors affecting its composition. J Path. Bact. 1965; 89: 95-122.

Songer, J.G. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 1996; 9: 216-234.

Spencer, R.J., Chesson, A. The effect of *Lactobacillus* spp. on the attachment of enterotoxigenic *Escherichia coli* to isolated porcine enterocytes. J. Appl. Bacteriol. 1994; 77: 215-220.

Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G.R., Collins, M.D., Doré, J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 1999; 65: 4799-4807.

Sylvester, F.A., Philpott, D., Gold, B., Lastovica, A., Forstner, J.F. Adherence to lipids and intestinal mucin by a recently recognized human pathogen, *Campylobacter upsaliensis*. Infect. Immun. 1996; 64: 4060-4066.

Szymanski, C.M., King, M., Haardt, M., Armstrong, G.D. *Campylobacter jejuni* motility and invasion of Caco-2 cells. Infect. Immun. 1995; 63: 4295-4300.

Tagg, J.R., Dierksen, K.P. Bacterial replacement therapy: adapting “germ warfare” to infection prevention. *Trends in Biotechnology*. 2003; 21: 217-223.

Tannock, G.W. 1999. The normal microflora: an introduction. *In*: Tannock, G.W., (ed), *Medical Importance of Normal Microflora*. Kluwer Academic Publishers, London, 1999: 1-23.

Todoriki K, Mukai T, Sato S, Toba T. Inhibition of adhesion of food-borne pathogens to Caco-2 cells by *Lactobacillus* strains. *J Appl Microbiol*. 2001; 91: 154-159

Toy, L.S., Mayer, L. Basic and clinical overview of the mucosal immune system. *Semin. Gastrointest. Dis.* 1996; 7: 2-11.

Thomas, W.E., Trintchina, E., Forero, M., Vogel, V., Sokurenko, E.V. Bacterial adhesion to target cells enhanced by shear force. *Cell*. 2002; 109: 913-23.

Tuomola, E.M., Ouwehand, A.C., Salminen, S.J. Human ileostomy glycoproteins as a model for small intestinal mucus to investigate adhesion of probiotics. *Lett. Appl. Microbiol*. 1999a; 28: 159-163.

Tuomola, E.M., Ouwehand, A.C., Salminen, S.J. The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol. Med. Microbiol*. 1999b; 26: 137-142.

Tuomola, E. *In vitro* adhesion of probiotic lactic acid bacteria. Thesis. Department of Biochemistry and Food Chemistry, University of Turku, Turku, 1999c.

Tuomola, E.M., Ouwehand, A.C., Salminen, S.J. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *Int J Food Microbiol.* 2000; 60: 75-81.

Twedt, D.C. *Clostridium perfringens*-associated enterotoxigenesis in dogs. *In*: Kirk, R.W., Bonagura, J.D., (eds), *Current veterinary therapy XI: small animal practice*. W.B. Saunders, Philadelphia, 1992: 602-604.

Uzzau, S., Leori, G.S., Petrucci, V., Watson, P.R., Schianchi, G., Bacciu, D., Mazzarello, V., Wallis, T.S., Rubino, S. *Salmonella enterica* serovar-host specificity does not correlate with the magnitude of intestinal invasion in sheep. *Infect. Immun.* 2001; 69: 3092-3099.

Vahjen W., Männer, K. The effect of a probiotic *Enterococcus faecium* product in diets of healthy dogs on bacteriological counts of *Salmonella* spp., *Campylobacter* spp. and *Clostridium* spp. in faeces. *Arch. Anim. Nutr.* 2003; 57: 229-233.

Vaughan, E.E., Schut, F., Heilig, H.G., Zoetendal, E.G., de Vos, W.M., Akkermans, A.D.
A molecular view of the intestinal ecosystem. Curr. Issues Intest. Microbiol. 2000; 1: 1-12.

Vaughan, E.E., de Vries, M.C., Zoetendal, E.G., Ben-Amor, K., Akkermans, A.D., de Vos, V.M. (2002) The intestinal LABs. Antonie van Leeuwenhoek, 82, 341-352.

Van Egmond M., Damen C.A., van Spriel A.B., Vidarsson G., van Garderen E., van de Winkel J.G. IgA and the IgA Fc receptor. Trends Immunol. 2001; 22: 205-211.

Verma, M., Davidson, E.A. Molecular cloning and sequencing of a canine tracheobronchial mucin cDNA containing a cysteine-rich domain. Proc. Natl. Acad. Sci. U S A. 1993; 90: 7144-7148.

Vigeant, M.A., Ford, R.M., Wagner, M, Tamm, L.K. Reversible and irreversible adhesion of motile *Escherichia coli* cells analyzed by total internal reflection aqueous fluorescence microscopy. Appl. Environ. Microbiol. 2002; 68: 2794-2801.

Wagner RD, Warner T, Roberts L, Farmer J, Balish E. Colonization of congenitally immunodeficient mice with probiotic bacteria. Infect Immun. 1997; 65 :3345-3351

Whitbread, T.J., Batt, R.M., Garthwaite, G. Relative deficiency of serum IgA in the German shepherd dog: A breed abnormality. Res. Vet. Sci. 1984; 37: 350-352.

Willard, M.D. Normal immune function of the gastrointestinal tract. Semin. Vet. Med. Surg. (Small Anim.). 1992; 7:107-111.

Wiström J., Norrby S.R., Myhre E.B., Eriksson S., Granström G., Lagergren L., Englund G., Nord C.E., Svenungsson B. Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. J. Antimicrob. Chemother. 2001; 47: 43-50.

WHO. The Medical Impact of the Use of Antimicrobials in Food Animals. Berlin, 13–17 October, 1997; WHO/EMC/ZOO/97.4.

Wilsson-Rahmberg, M., Jonsson, O. Method for long-term intestinal access in the dog. Lab. Anim. 1997; 31: 231-240.

Wooldridge, K.G., Ketley, J.M. *Campylobacter* – host cell interactions. Trends Microbiol. 1997; 5: 96-102.

Wu, H.Y., Russel, M.W. Induction of mucosal and systemic immune responses by intranasal immunization using recombinant cholera toxin B subunit as adjuvant. Vaccine. 1998; 16: 286-292.

Xu, J., Gordon, J.I. Honor thy symbionts. Proc. Natl. Acad. Sci. 2003; 100: 10452-10459.

Yamada M, Saito T, Toba T, Kitazawa H, Uemura J, Itoh T. Hemagglutination activity of *Lactobacillus acidophilus* group lactic acid bacteria. Biosci Biotechnol Biochem. 1994; 58: 910-915.

Yasui H, Shida K, Matsuzaki T, Yokokura T. Immunomodulatory function of lactic acid bacteria. Antonie Van Leeuwenhoek. 1999; 76: 383-389.

Zentek, J., Molitor, D., Kamphues, J. *Enterococcus faecium* as probiotic feed additive in dogs. Kleintierpraxis. 1998; 43: 187-197.